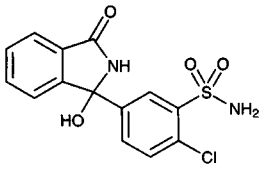
Chlorthalidone



Molecular formula: $C_{14}H_{11}ClN_2O_4S$
Molecular weight: 338.77
CAS Registry No.: 77-36-1
Merck Index: 2246
Lednicer No.: 1 322

SAMPLE

Matrix: blood
Sample preparation: 1 mL Plasma + 250 ng xipamide, mix for 10 s, add 10 mL dichloromethane:2-propanol 75:25, shake for 10 min. Centrifuge at 2000 g for 10 min at 4°. Remove the organic phase and evaporate it to dryness under a stream of nitrogen at 50°. Reconstitute the residue in 200 µL mobile phase , mix for 10 s. Centrifuge at 6500 g for 10 min. Inject a 40 µL aliquot of the supernatant.

HPLC VARIABLES

Guard column: 4 × 4 5 µm LiChrospher 100 RP-18
Column: 250 × 4 5 µm Supelcosil LC-18 (Supelco)
Mobile phase: n-Propanol:buffer 5:95 (Buffer was 50 mM sodium dodecyl sulfate in 10 mM pH 5.8 sodium phosphate buffer.)
Flow rate: 1.3
Injection volume: 40
Detector: UV 225

CHROMATOGRAM

Retention time: 6.05
Internal standard: xipamide (8.58)
Limit of quantitation: 10 ng/mL

OTHER SUBSTANCES

Extracted: albuterol, atenolol

KEY WORDS

plasma; pharmacokinetics

REFERENCE

Giachetti,C.; Tenconi,A.; Canali,S.; Zanolo,G. Simultaneous determination of atenolol and chlorthalidone in plasma by high-performance liquid chromatography. Application to pharmacokinetic studies in man, *J.Chromatogr.B*, **1997**, *698*, 187–194.

SAMPLE

Matrix: formulations
Sample preparation: Grind 2 tablets, sonicate in 15 mL water for 15 min, shake vigorously for 30 min, add 25 mL MeOH, shake 1 h, dilute to 50 mL with MeOH, mix, centrifuge, filter (1 µm or smaller), inject 25 µL aliquot

HPLC VARIABLES

Column: 250 × 4.6 trimethylsilyl chloride bonded to 5-6 µm spherical silica
Mobile phase: MeOH:buffer 65:35 (Buffer was 2.2 mM KH_2PO_4 + 16 mM Na_2HPO_4, pH 7.9.)
Flow rate: 1
Injection volume: 25
Detector: UV 254

CHROMATOGRAM
Retention time: 7.5

OTHER SUBSTANCES
Simultaneous: clonidine

KEY WORDS
tablets

REFERENCE
Walters,S.M.; Stonys,D.B. Determination of chlorthalidone and clonidine hydrochloride in tablets by HPLC, *J.Chromatogr.Sci.*, **1983**, *21*, 43–45.

SAMPLE
Matrix: solutions

HPLC VARIABLES
Column: 250 × 4 5 μm ChiraDex, LichroCART
Mobile phase: MeOH:water 20:80 (A) or MeOH:pH 4.1 triethylammonium acetate buffer 2:98 (B)
Column temperature: 6 (A), 45 (B)
Flow rate: 1 (A) or 0.8 (B)
Detector: UV 220

CHROMATOGRAM
Retention time: 23.51 (enantiomer I, A), 37.60 (enantiomer II, A), 15.44 (enantiomer I, B), 20.67 (enantiomer II, B)

KEY WORDS
chiral

REFERENCE
Cabrera,K.; Jung,M.; Fluck,M.; Schurig,V. Determination of enantiomerization barriers by computer simulation of experimental elution profiles obtained by high-performance liquid chromatography on a chiral stationary phase, *J.Chromatogr.A*, **1996**, *731*, 315–321.

SAMPLE
Matrix: solutions

HPLC VARIABLES
Column: 250 × 4.6 5 μm Supelcosil LC-DP (A) or 250 × 4 5 μm LiChrospher 100 RP-8 (B)
Mobile phase: MeCN:0.025% phosphoric acid:buffer 25:10:5 (A) or 60:25:15 (B) (Buffer was 9 mL concentrated phosphoric acid and 10 mL triethylamine in 900 mL water, adjust pH to 3.4 with dilute phosphoric acid, make up to 1 L.)
Flow rate: 0.6
Injection volume: 25
Detector: UV 229

CHROMATOGRAM
Retention time: 5.14 (A), 4.03 (B)

OTHER SUBSTANCES

Also analyzed: acebutolol, acepromazine, acetaminophen, acetazolamide, acetophenazine, albuterol, alprazolam, amitriptyline, amobarbital, amoxapine, antipyrine, atenolol, atropine, azatadine, baclofen, benzocaine, bromocriptine, brompheniramine, brotizolam, bupivacaine, buspirone, butabarbital, butalbital, caffeine, carbamazepine, cetirizine, chlorcyclizine, chlordiazepoxide, chlormezanone, chloroquine, chlorpheniramine, chlorpromazine, chlorpropamide, chlorprothixene, chlorzoxazone, cimetidine, cisapride, clomipramine, clonazepam, clonidine, clozapine, cocaine, codeine, colchicine, cyclizine, cyclobenzaprine, dantrolene, desipramine, diazepam, diclofenac, diflunisal, diltiazem, diphenhydramine, diphenidol, diphenoxylate, dipyridamole, disopyramide, dobutamine, doxapram, doxepin, droperidol, encainide, ethidium bromide, ethopropazine, fenoprofen, fentanyl, flavoxate, fluoxetine, fluphenazine, flurazepam, flurbiprofen, fluvoxamine, furosemide, glutethimide, glyburide, guaifenesin, haloperidol, homatropine, hydralazine, hydrochlorothiazide, hydrocodone, hydromorphone, hydroxychloroquine, hydroxyzine, ibuprofen, imipramine, indomethacin, ketoconazole, ketoprofen, ketorolac, labetalol, levorphanol, lidocaine, loratadine, lorazepam, lovastatin, loxapine, mazindol, mefenamic acid, meperidine, mephenytoin, mepivacaine, mesoridazine, metaproterenol, methadone, methdilazine, methocarbamol, methotrexate, methotrimeprazine, methoxamine, methyldopa, methylphenidate, metoclopramide, metolazone, metoprolol, metronidazole, midazolam, moclobemide, morphine, nadolol, nalbuphine, naloxone, naphazoline, naproxen, nifedipine, nizatidine, norepinephrine, nortriptyline, oxazepam, oxycodone, oxymetazoline, paroxetine, pemoline, pentazocine, pentobarbital, pentoxifylline, perphenazine, pheniramine, phenobarbital, phenol, phenolphthalein, phentolamine, phenylbutazone, phenyltoloxamine, phenytoin, pimozide, pindolol, piroxicam, pramoxine, prazepam, prazosin, probenecid, procainamide, procaine, prochlorperazine, procyclidine, promazine, promethazine, propafenone, propantheline, propiomazine, propofol, propranolol, protriptyline, quazepam, quinidine, quinine, racemethorphan, ranitidine, remoxipride, risperidone, salicylic acid, scopolamine, secobarbital, sertraline, sotalol, spironolactone, sulfinpyrazone, sulindac, temazepam, terbutaline, terfenadine, tetracaine, theophylline, thiethylperazine, thiopental, thioridazine, thiothixene, timolol, tocainide, tolbutamide, tolmetin, trazodone, triamterene, triazolam, trifluoperazine, triflupromazine, trimeprazine, trimethoprim, trimipramine, verapamil, warfarin, xylometazoline, yohimbine, zopiclone

KEY WORDS

also details of plasma extraction

REFERENCE

Koves,E.M. Use of high-performance liquid chromatography-diode array detection in forensic toxicology, *J.Chromatogr.A*, **1995**, *692*, 103–119.

SAMPLE

Matrix: solutions

Sample preparation: Prepare a solution in MeOH:water 80:20, inject a 6 μL aliquot.

HPLC VARIABLES

Guard column: 5 × 4 10 μm LiChrosorb RP-8

Column: 100 × 4.6 5 μm Spheri RP-18 (Brownlee)

Mobile phase: MeOH:water 80:20 containing 2 g/L lithium perchlorate

Flow rate: 0.5

Injection volume: 6

Detector: E, ESA Model 5100A Coulochem, model 5020 guard cell +950 mV, Model 5010 analytical cell + 400 mV, palladium reference electrode, following post-column photolysis. The effluent from the column flowed through a 20 m × 0.3 mm coil of PTFE tubing and was irradiated at 254 nm with a Sylvania GTE 8 W low-pressure lamp.

CHROMATOGRAM

Limit of detection: 267 ng/mL

OTHER SUBSTANCES
Also analyzed: bendroflumethiazide, butizide, ethacrynic acid, furosemide, hydrochlorothiazide

KEY WORDS
post-column reaction

REFERENCE
Macher,M.; Wintersteiger,R. Improved electrochemical detection of diuretics in high-performance liquid chromatographic analysis by postcolumn on-line photolysis, *J.Chromatogr.A*, **1995**, *709*, 257–264.

SAMPLE
Matrix: solutions
Sample preparation: Inject a 20 μL aliquot of an 8 μg/mL solution.

HPLC VARIABLES
Column: 250 × 4 4 μm Superspher 100 RP-18
Mobile phase: EtOH:buffer 20:80 containing 25 mM β-cyclodextrin substituted with 2-hydroxy-3-trimethylammoniumpropyl groups (Roquette Frères, Lestrem, France) (Buffer was 0.8% triethylamine adjusted to pH 4.1 with acetic acid.)
Column temperature: 22.5
Flow rate: 0.8
Injection volume: 20
Detector: UV 254

CHROMATOGRAM
Retention time: 11.26 (+), 12.31 (-)

KEY WORDS
chiral

REFERENCE
Roussel,C.; Favrou,A. Cationic β-cyclodextrin: a new versatile chiral additive for separation of drug enantiomers by high-performance liquid chromatography, *J.Chromatogr.A*, **1995**, *704*, 67–74.

SAMPLE
Matrix: urine
Sample preparation: Inject 50 μL untreated urine onto column A and elute to waste with mobile phase A, after 4 min elute the contents of column A onto column B with mobile phase B, monitor the effluent from column B. After another 5 min re-equilibrate column A with mobile phase A and column B with mobile phase B.

HPLC VARIABLES
Column: A 20 × 2.1 30 μmHypersil ODS C18; B 250 × 4 5 μm LiChroCART ChiraDex (Merck)
Mobile phase: A Water; B MeOH:50 mM pH 4 acetate buffer 40:60 (Prepare buffer by adding 500 μL propylamine hydrochloride to 500 mL water, dissolve 2 g sodium acetate, adjust pH to 4 with glacial acetic acid.) (Mobile phase B is also given as MeCN:buffer 40:60 and MeOH:buffer 60:40.)
Flow rate: 1
Injection volume: 50
Detector: UV 230

CHROMATOGRAM
Retention time: 7.8, 8.2 (enantiomers)
Limit of detection: 20ng/mL
Limit of quantitation: 250 ng/mL

KEY WORDS
chiral; direct injection; column-switching; pharmacokinetics

REFERENCE
Herráez-Hernández,R.; Campíns-Falco,P.; Sevillano-Cabeza,A. Application of column switching in high-performance liquid chromatographic analysis of chlorthalidone enantiomers in untreated urine, *J.Liq.Chromatogr.Rel.Technol.*, **1996**, *19*, 403–414.

SAMPLE
Matrix: urine
Sample preparation: Add 400 μL 3 μg/mL IS in water to 600 μL urine, inject a 50 μL aliquot onto column A at 1 mL/min. Elute to waste with 2 mL water, after 2 min backflush the contents of column A onto column B with mobile phase, after 30 s remove column A from the circuit, elute column B with mobile phase, monitor the effluent from column B. Wash column A with MeCN:water 95:5 and equilibrate it with water.

HPLC VARIABLES
Column: A 12.5 × 4 5 μm Stable Bond-CN (Zorbax); B 250 × 4.6 5 μm Ultrasphere C18
Mobile phase: MeCN:10 mM pH 7.0 phosphate buffer 20:80
Flow rate: 2
Injection volume: 50
Detector: UV 214

CHROMATOGRAM
Retention time: 9.8
Internal standard: 2,7-dihydroxynaphthalene (11.2)
Limit of detection: 20 ng/mL
Limit of quantitation: 100 ng/mL

OTHER SUBSTANCES
Simultaneous: 2-(3-aminosulfonyl-4-chlorbenzoyl) benzoic acid, caffeine
Noninterfering: methyldopa, salicylic acid
Interfering: naproxen

KEY WORDS
column-switching; pharmacokinetics

REFERENCE
Salado,S.C.; Vera-Avila,L.E. On-line solid-phase extraction and high-performance liquid chromatographic determination of chlorthalidone in urine, *J.Chromatogr.B*, **1997**, *690*, 195–202.

SAMPLE
Matrix: urine
Sample preparation: 2 mL Urine + 2 mL 1 M pH 4.1 NaH_2PO_4 + 4 mL ethyl acetate, vortex for 2 min, centrifuge at 1500 g for 5 min. Remove the organic phase and add it to 5 mL 100 mM pH 7.5 Na_2HPO_4, vortex for 2 min, centrifuge at 1500 g for 5 min. Remove the organic layer and evaporate it to dryness under a stream of nitrogen at 60°, reconstitute the residue in 100 μL MeCN:10 mM pH 3.0 phosphate buffer, inject a 5 μL aliquot.

HPLC VARIABLES
Column: 125 × 4 5 μm LiCHrosorb RP-18
Mobile phase: Gradient. MeCN:10 mM pH 3.0 phosphate buffer 10:90 for 1.5 min then to 35:65 over 2 min
Column temperature: 50
Flow rate: 1.5
Injection volume: 5
Detector: UV 271

CHROMATOGRAM

Retention time: 4.1
Limit of quantitation: 1000 ng/mL

OTHER SUBSTANCES

Extracted: chlorothiazide, hydrochlorothiazide, quinethazone, methyclothiazide, clopamide, furosemide, metolazone, mefruside, bendroflumethiazide, cyclopenthiazide, bumetanide
Simultaneous: indapamide, clorexolone, ethacrynic acid
Noninterfering: aspirin, albuterol, allopurinol, alprenolol, atenolol, captopril, carbimazole, clonidine, coloxyl, danthron, diazepam, digoxin, doxepin, glibenclamide, hydralazine, indomethacin, labetalol, metformin, methyldopa, metoprolol, mianserin, minoxidil, nifedipine, nitrazepam, oxazepam, oxprenolol, pindolol, prazosin, propranolol, senokot, theophylline, trifluoperazine

REFERENCE

Fullinfaw,R.O.; Bury,R.W.; Moulds,R.F.W. Liquid chromatographic screening of diuretics in urine, *J.Chromatogr.*, **1987**, *415*, 347–356.

SAMPLE

Matrix: urine
Sample preparation: 2 mL Urine + 0.5 g solid buffer I (pH 5-5.5), vortex 15 s, add 4 mL ethyl acetate, agitate for 10 min, centrifuge at 600 g for 5 min. Remove organic layer and vortex it with 2 mL 5% aqueous lead acetate for 10 s, centrifuge at 600 g for 5 min, remove and keep organic phase. 2 mL Urine + 0.5 g solid buffer II (pH 9-9.5), vortex 15 s, add 4 mL ethyl acetate, agitate for 10 min, centrifuge at 600 g for 5 min. Remove organic layer and combine it with previous organic layer. Evaporate to dryness at 50° under a stream of nitrogen, reconstitute in 300 μL 50 μg/mL β-hydroxyethyltheophylline in MeOH, inject 5 μL aliquot. (Solid buffer I was KH_2PO_4:Na_2HPO_4 99:1, solid buffer II was $NaHCO_3$:K_2CO_3 3:2.)

HPLC VARIABLES

Column: 250 × 4.6 5 μm HP Hypersil ODS (A) or HP LiChrosorb RP-18 (B)
Mobile phase: Gradient. MeCN:buffer from 15:85 at 2 min to 80:20 at 20 min (Buffer was 50 mM NaH_2PO_4 containing 16 mM propylamine hydrochloride, adjusted to pH 3 with concentrated phosphoric acid.)
Flow rate: 1
Injection volume: 5
Detector: UV 230, UV 275

CHROMATOGRAM

Retention time: 9.0 (A), 9.8 (B)
Internal standard: β-hydroxyethyltheophylline (3.7 (A), 4.4 (B))
Limit of detection: 1000 ng/mL

OTHER SUBSTANCES

Extracted: furosemide, metolazone, amiloride, acetazolamide, chlorothiazide, hydrochlorothiazide, quinethazone, triamterene, hydroflumethiazide, dichlorphenamide, trichloromethiazide, methyclothiazide, benzthiazide, cyclothiazide, polythiazide, bendroflumethiazide, ethacrynic acid, bumetanide, probenecid, spironolactone, canrenone, flumethiazide
Noninterfering: acetaminophen, aspirin, caffeine, diflunisal, fenoprofen, ibuprofen, indomethacin, methocarbamol, naproxen, phenylbutazone, sulindac, tetracycline, theobromine, theophylline, tolmetin, trimethoprim, verapamil

REFERENCE

Cooper,S.F.; Massé,R.; Dugal,R. Comprehensive screening procedure for diuretics in urine by high-performance liquid chromatography, *J.Chromatogr.*, **1989**, *489*, 65–88.

SAMPLE
Matrix: urine
Sample preparation: Make 5 mL urine alkaline (pH 9-10), add 2 g NaCl, extract twice with 6 mL ethyl acetate. Combine the organic layers and evaporate them to dryness under a stream of nitrogen, reconstitute the residue in 200 μL MeCN/water, inject a 10-20 μL aliquot.

HPLC VARIABLES
Column: 100 × 4 5 μm SGE 100 GL-4 C18P (Scientific Glass Engineering)
Mobile phase: MeCN:MeOH:water:trifluoroacetic acid 4.5:10.5:85:0.5
Flow rate: 0.8 or 1
Injection volume: 10-20
Detector: MS, ZAB2-SEQ (VG), PSP source coupled to LC, source 250°, probe 240-260°, scan m/z 200-550 or UV 270

CHROMATOGRAM
Retention time: 2.5
Limit of detection: 100 ng (by MS)

OTHER SUBSTANCES
Extracted: amiloride, triamterene, furosemide, benzthiazide, bendroflumethiazide

REFERENCE
Ventura,R.; Fraisse,D.; Becchi,M.; Paisse,O.; Segura,J. Approach to the analysis of diuretics and masking agents by high-performance liquid chromatography-mass spectrometry in doping control, *J.Chromatogr.*, **1991**, *562*, 723–736.

SAMPLE
Matrix: urine
Sample preparation: Direct injection.

HPLC VARIABLES
Guard column: 35 × 4 5 μm Spherisorb ODS-2
Column: 120 × 4 5 μm Spherisorb ODS-2
Mobile phase: MeOH:50 mM sodium dodecyl sulfate 5:95
Column temperature: 50
Flow rate: 1
Injection volume: 20
Detector: UV 224

CHROMATOGRAM
Retention time: 8.8
Limit of detection: 500 ng/mL

OTHER SUBSTANCES
Simultaneous: bendroflumethiazide

REFERENCE
Bonet Domingo,E.; Medina Hernández,M.J.; Ramis Ramos,G.; Garcia Alvarez-Coque,M.C. High-performance liquid chromatographic determination of diuretics in urine by micellar liquid chromatography, *J.Chromatogr.*, **1992**, *582*, 189–194.

SAMPLE
Matrix: urine
Sample preparation: Condition a 1 mL 100 mg Bond-Elut C8 SPE cartridge with 500 μL MeOH and 500 μL water. 2 mL urine + 300 μL 1 μg/mL triamterene in MeOH, add to

the SPE cartridge, wash with 500 μL water, elute with 500 μL MeOH, filter (0.45 μm) the eluate, inject a 5 μL aliquot.

HPLC VARIABLES
Column: 125 × 4 5 μm HP-LiChrospher 100 RP 18
Mobile phase: Gradient. MeCN:buffer from 0:100 to 30:70 over 5 min, maintain at 30:70. (Buffer was 3.45 g $NaH_2PO_4.H_2O$ + 700 μL propylamine hydrochloride in 500 mL water, adjust pH to 3 with concentrated phosphoric acid.)
Flow rate: 1
Injection volume: 5
Detector: UV 230

CHROMATOGRAM
Retention time: 6.1
Internal standard: triamterene (3.8)
Limit of detection: 6 ng/mL

OTHER SUBSTANCES
Simultaneous: atenolol, oxprenolol, reserpine, spironolactone
Noninterfering: metabolites

KEY WORDS
SPE

REFERENCE
Campíns-Falcó,P.; Herráez-Hernández,R.; Sevillano-Cabeza,A. Simple and sensitive reversed-phase liquid chromatographic assay for analysis of chlorthalidone in urine, *J.Liq.Chromatogr.*, **1993**, *16*, 2571–2581.

SAMPLE
Matrix: urine
Sample preparation: Buffer urine to 4.9 by mixing with an equal volume of pH 4.9 200 mM sodium phosphate buffer. Inject a 40 μL aliquot onto column A with mobile phase A, after 3 min backflush the contents of column A onto column B with mobile phase B and start the gradient. At the end of the run re-equilibrate for 10 min.

HPLC VARIABLES
Column: A 20 × 4 5 μm Hypersil octadecylsilica ODS; B 200 × 4.6 5 μm Shiseido SG-120 polymer-based C18
Mobile phase: A water; B Gradient. MeCN:buffer from 7:93 to 15:85 over 3.5 min, to 50:50 over 8.5 min, maintain at 50:50 for 11 min (Buffer was 6.9 g $NaH_2PO_4.H_2O$ in 1 L water, pH adjusted to 3.1 with phosphoric acid.)
Flow rate: 1
Injection volume: 40
Detector: UV 230

CHROMATOGRAM
Retention time: 13.7
Limit of detection: 1000 ng/mL

OTHER SUBSTANCES
Extracted: acetazolamide, amiloride, bendroflumethiazide, benzthiazide, bumetanide, caffeine, carbamazepine, chlorothiazide, clopamide, dichlorfenamide, ethacrynic acid, furosemide, hydrochlorothiazide, metyrapone, probenecid, spironolactone, triamterene, trichlormethiazide

KEY WORDS
column-switching; optimum detection wavelengths vary for each drug

REFERENCE

Saarinen,M.; Sirén,H.; Riekkola,M.-L. A column switching technique for the screening of diuretics in urine by high performance liquid chromatography, *J.Liq.Chromatogr.*, **1993**, *16*, 4063–4078.

SAMPLE

Matrix: urine

Sample preparation: 5 mL Urine + 50 μL 100 μg/mL 7-propyltheophylline in MeOH + 200 μL ammonium chloride buffer + 2 g NaCl, extract with 6 mL ethyl acetate by rocking at 40 movements/min for 20 min and centrifuging at 800 g for 5 min, repeat extraction, combine organic layers, evaporate to dryness at 40° under a stream of nitrogen. Reconstitute in 200 μL MeCN:water 15:85 and inject 20 μL aliquots. (Ammonium chloride buffer was 28 g ammonium chloride in 100 mL water with the pH adjusted to 9.5 with concentrated ammonia solution.)

HPLC VARIABLES

Column: 75 × 4.6 3 μm Ultrasphere ODS

Mobile phase: Gradient. MeCN:100 mM ammonium acetate adjusted to pH 3 with concentrated phosphoric acid. From 10:90 to 15:85 over 2 min to 55:45 over 3 min to 60:40 over 3 min. Kept at 60:40 for 1 min, decreased to 10:90 over 1 min and equilibrated at 10:90 for 2 min.

Flow rate: 1

Injection volume: 20

Detector: UV 270

CHROMATOGRAM

Retention time: 4.7

Internal standard: 7-propyltheophylline (4.5)

Limit of detection: 200 ng/mL

OTHER SUBSTANCES

Simultaneous: xipamide, bumetanide, acetazolamide, amiloride, bendroflumethiazide, buthiazide, benzthiazide, canrenone, caffeine, clopamide, cyclothiazide, ethacrynic acid, furosemide, hydrochlorothiazide, mesocarb, piretanide, polythiazide, probenecid, spironolactone, torsemide, triamterene

Interfering: morazone, diclofenamide

REFERENCE

Ventura,R.; Nadal,T.; Alcalde,P.; Pascual,J.A.; Segura,J. Fast screening method for diuretics, probenecid and other compounds of doping interest, *J.Chromatogr.A*, **1993**, *655*, 233–242.

SAMPLE

Matrix: urine

Sample preparation: Direct injection into column A with mobile phase A for 1 min then back flush onto column B with mobile phase B.

HPLC VARIABLES

Column: A 20 × 2.1 30 μm Hypersil ODS-C18; B 250 × 4 Hypersil ODS-C18

Mobile phase: A Water; B Gradient. MeCN:buffer 15:85 for 1.5 min then to 80:20 over 8 min. Keep at 80:20 for 2.5 min then re-equilibrate with 15:85. (Buffer was 50 mM NaH_2PO_4 + 1.4 mL propylamine hydrochloride per liter adjusted to pH 3 with concentrated phosphoric acid.)

Flow rate: 1

Injection volume: 50

Detector: UV 230

CHROMATOGRAM

Retention time: 7.5

Limit of detection: 4 ng/mL.

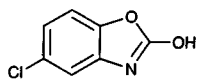
OTHER SUBSTANCES

Simultaneous: bumetanide, ethacrynic acid, acetazolamide, amiloride, bendroflumethiazide, cyclothiazide, furosemide, hydrochlorothiazide, probenecid, spironolactone, triamterene

REFERENCE

Campíns-Falco,P.; Herráez-Hernández,R.; Sevillano-Cabeza,A. Column-switching techniques for screening of diuretics and probenecid in urine samples, *Anal.Chem.*, **1994**, *66*, 244–248.

Chlorzoxazone



Molecular formula: $C_7H_4ClNO_2$
Molecular weight: 169.57
CAS Registry No.: 95-25-0
Merck Index: 2249
Lednicer No.: 1 323

SAMPLE

Matrix: blood
Sample preparation: 500 μL Serum + 1 mL 200 mM pH 4.75 sodium acetate buffer + 500 μL 0.2% sodium chloride containing 1000 U β-glucuronidase, vortex, incubate at 37° for 3 h. Add 100 μL 20 μg/mL IS, 400 μL 10% perchloric acid and 4 mL ethyl acetate, vortex for 5 min, centrifuge at 1200 g for 5 min. Remove the organic layer and evaporate it under a gentle stream of nitrogen at 40°. Reconstitute the residue in 50 μL mobile phase, inject a 10 μL aliquot.

HPLC VARIABLES

Column: 100 × 4.6 2 μm TSK gel Super-ODS (A) or 100 × 4.6 5 μm Hypersil ODS (B)
Mobile phase: MeCN:0.15% pH 5.0 ammonium acetate 10:90
Flow rate: 0.3
Injection volume: 10
Detector: UV 287

CHROMATOGRAM

Retention time: 7 (A), 11 (B)
Internal standard: 5-chloro-2-methylbenzoxazole (12 (A); 19 (B))
Limit of quantitation: 10 ng/mL (A)

OTHER SUBSTANCES

Extracted: metabolites

KEY WORDS

serum; pharmacokinetics

REFERENCE

Tanaka,E. Simultaneous determination of chlorzoxazone, indicator of CYP2E1, and its metabolite in human serum using a new reversed-phase chromatographic column of 2-μm porous microspherical silica-gel, *J.Pharm.Biomed.Anal.*, **1998**, *16*, 899–904.

SAMPLE

Matrix: blood
Sample preparation: Evaporate 100 μL 100 μg/mL phenacetin in MeOH into the bottom of a tube with a stream of nitrogen, add 1 mL plasma, vortex for 1 min, add 1 mL 50 mM sulfuric acid, add four 125 mg portions of ammonium sulfate with vortexing and heating on a steam bath after each addition, add 5 mL ether, vortex, centrifuge at 5000 rpm for 5 min, freeze in dry ice/acetone. Remove the organic layer and filter (0.5 μm) it, evaporate to dryness under a stream of nitrogen on a steam bath, reconstitute the residue in 100 μL MeOH, inject a 20 μL aliquot.

HPLC VARIABLES

Column: 10 μm μBondapak C18
Mobile phase: MeOH:water 40:60
Flow rate: 2
Injection volume: 20

Detector: UV 280

CHROMATOGRAM
Retention time: 8.5
Internal standard: phenacetin (6)
Limit of detection: 80 ng

OTHER SUBSTANCES
Extracted: metabolites
Noninterfering: acetaminophen

KEY WORDS
plasma

REFERENCE
Honigberg,I.L.; Stewart,J.T.; Coldren,J.W. Liquid chromatography in pharmaceutical analysis X: Determination of chlorzoxazone and hydroxy metabolite in plasma, *J.Pharm.Sci.*, **1979**, *68*, 253–255.

SAMPLE
Matrix: blood
Sample preparation: Condition a 3 mL 200 mg Bond Elut C18 SPE cartridge with one volume of MeOH and one volume of 50 μL/L glacial acetic acid in water. 500 μL Plasma + 10 μL 40 μg/mL 5-fluorobenzoxazolone in MeOH + 1 mL 200 mM pH 4.75 sodium acetate buffer + 500 μL 0.2% NaCl containing 1000 U β-glucuronidase (Type G0751, Sigma), vortex, heat at 37° for 3 h, add to SPE cartridge, wash with 1 mL 50 μL/L glacial acetic acid in water, elute with two 500 μL aliquots of MeCN. Evaporate the eluate to dryness under a stream of nitrogen at 40°, reconstitute the residue in 250 μL MeCN:100 mM ammonium acetate 40:60, vortex, centrifuge at 13600 g for 1 min, inject a 50 μL aliquot of the supernatant.

HPLC VARIABLES
Guard column: 20 × 2 pellicular C18
Column: 300 × 3.9 10 μm μBondapak C18
Mobile phase: MeCN:THF:100 mM pH 7.0 ammonium acetate 22.5:5.5:72
Flow rate: 1
Injection volume: 50
Detector: UV 283

CHROMATOGRAM
Retention time: 17.65
Internal standard: 5-fluorobenzoxazolone (10.53)
Limit of quantitation: 100 ng/mL

OTHER SUBSTANCES
Extracted: metabolites

KEY WORDS
plasma; pharmacokinetics; SPE

REFERENCE
Stiff,D.D.; Frye,R.F.; Branch,R.A. Sensitive high-performance liquid chromatographic determination of chlorzoxazone and 6-hydroxychlorzoxazone in plasma, *J.Chromatogr.*, **1993**, *613*, 127–131.

SAMPLE
Matrix: blood
Sample preparation: 500 μL Whole blood or plasma + 25 μL 250 μg/mL p-acetophenetide, vortex, add 2 mL MeCN, vortex, centrifuge at high speed for 5 min. Remove the MeCN

layer and evaporate it to dryness under a stream of nitrogen, reconstitute the residue in 200 μL mobile phase, vortex for 10 s, inject a 50 μL aliquot.

HPLC VARIABLES
Column: 250 × 4.6 10 μm Econosil C18
Mobile phase: MeOH:water 40:60
Flow rate: 2
Injection volume: 50
Detector: UV (wavelength not given)

CHROMATOGRAM
Internal standard: p-acetophenetide
Limit of detection: 4 ng/mL

KEY WORDS
pharmacokinetics; whole blood; plasma

REFERENCE
de Vries,J.D.; Salphati,L.; Horie,S.; Becker,C.E.; Hoener,B.A. Variability in the disposition of chlorzoxazone, *Biopharm.Drug Dispos.*, **1994**, *15*, 587–597.

SAMPLE
Matrix: blood, urine
Sample preparation: Plasma. 500 μL Plasma + 1 mL 200 mM pH 4.75 sodium acetate buffer + 1000 U β-glucuronidase in 500 μL 0.2% NaCl, vortex, incubate at 37° for 3 h. Add 5 mL diethyl ether, shake for 10 min, centrifuge at 2000 g for 10 min. Evaporate the organic layer under a stream of nitrogen at 40°. Reconstitute the residue in 200 μL mobile phase, inject a 50 μL aliquot. Urine. Dilute sample 1:1 (v/v) with water. 500 μL Diluted urine + 1 mL 200 mM pH 4.75 sodium acetate buffer + 1000 U β-glucuronidase in 500 μL 0.2% NaCl, vortex, incubate at 37° for 3 h. Add 5 mL diethyl ether, shake for 10 min, centrifuge at 2000 g for 10 min. Evaporate the organic layer under a stream of nitrogen at 40°. Reconstitute the residue in 500 μL mobile phase, inject a 50 μL aliquot.

HPLC VARIABLES
Guard column: 20 × 2 pellicular C18 (Alltech)
Column: 300 × 3.9 10 μm Alphabond C18 (Alltech) (plasma) or 300 × 3.9 10 μm μBondapak C18 (urine)
Mobile phase: MeCN:THF:100 mM ammonium acetate 22.5:5.5:72
Flow rate: 1.0
Injection volume: 50
Detector: UV 283

CHROMATOGRAM
Retention time: 17.5 (plasma)
Internal standard: 3-aminophenyl sulfone (plasma, 12.1), phenacetin (urine, 10.9)
Limit of quantitation: 100 ng/mL (plasma)

OTHER SUBSTANCES
Extracted: metabolites

KEY WORDS
plasma

REFERENCE
Frye,R.F.; Stiff,D.D. Determination of chlorzoxazone and 6-hydroxychlorzoxazone in human plasma and urine by high-performance liquid chromatography, *J.Chromatogr.B*, **1996**, *686*, 291–296.

SAMPLE
Matrix: blood, urine
Sample preparation: Dilute urine 1:100. 500 μL Serum or diluted urine + 500 μL 2 M pH 4.5 acetate buffer + 20 μL H. pomatia juice, heat at 37° overnight, add 4 mL 600 mM perchloric acid, centrifuge at 3500 g for 10 min. Remove the supernatant and add it to 4 mL ethyl acetate, shake for 10 min, centrifuge at 4° for 10 min, repeat the extraction. Combine the organic layers and evaporate them to dryness under a stream of nitrogen, reconstitute the residue in 250 μL mobile phase, inject a 20 μL aliquot. (H. pomatia juice from IBF Biotechnics contained 100000 Fishman U/mL β-glucuronidase and 1000000 Roy U/mL sulfatase.)

HPLC VARIABLES
Column: 250 × 4.6 5μm Nucleosil ODS
Mobile phase: MeCN:0.5% acetic acid 30:70
Flow rate: 1
Injection volume: 20
Detector: UV 287

CHROMATOGRAM
Retention time: 17
Limit of detection: 50 ng/mL
Limit of quantitation: 500 ng/mL

OTHER SUBSTANCES
Extracted: metabolites

KEY WORDS
serum; pharmacokinetics

REFERENCE
Lucas,D.; Berthou,F.; Girre,C.; Poitrenaud,F.; Ménez,J.-F. High-performance liquid chromatographic determination of chlorzoxazone and 6-hydroxychlorzoxazone in serum: a tool for indirect evaluation of cytochrome P4502E1 activity in humans, *J.Chromatogr.*, **1993**, *622*, 79–86.

SAMPLE
Matrix: microsomal incubations
Sample preparation: Add 200 μL 1.5 μg/mL phenacetin in MeCN to 400 μL microsomal incubation, vortex for 30 s, centrifuge at 10000 g for 4 min. Add 2 mL diethyl ether to the supernatant, vortex for 30 s, centrifuge at 2000 g for 1 min, dry the ether layer under vacuum, reconstitute the residue with 150 μL mobile phase, vortex, inject a 100 μL aliquot.

HPLC VARIABLES
Column: 100 × 4.6 Brownlee Spheri-5 C8
Mobile phase: MeCN:0.5% phosphoric acid 30:70
Flow rate: 1
Injection volume: 100
Detector: UV 287

CHROMATOGRAM
Retention time: 6.4
Internal standard: phenacetin (4.3)

OTHER SUBSTANCES
Extracted: 6-hydroxychlorzoxazone

KEY WORDS
liver; rat

REFERENCE

Chittur,S.V.; Tracy,T.S. Rapid and sensitive high-performance liquid chromatographic assay for 6-hydroxychlorzoxazone and chlorzoxazone in liver microsomes, *J.Chromatogr.B*, **1997**, *693*, 479–483.

SAMPLE

Matrix: microsomal incubations

Sample preparation: 1 mL Microsomal incubation + 5 mL ethyl acetate + umbelliferone + 50 μL 42.5% phosphoric acid, vortex, centrifuge. Remove 4 mL of the organic layer and evaporate it to dryness under a stream of nitrogen, reconstitute the residue in 200 μL mobile phase, inject an aliquot.

HPLC VARIABLES

Column: 150 × 4.6 5 μm C18 (Supelco)
Mobile phase: Gradient. MeCN:0.25% acetic acid 21:79 for 11 min, to 95:5 over 7 min.
Flow rate: 1
Detector: UV 296

CHROMATOGRAM

Retention time: 11.54
Internal standard: umbelliferone (4.76)

OTHER SUBSTANCES

Extracted: metabolites

KEY WORDS

rat; liver

REFERENCE

Jayyosi,Z.; Knoble,D.; Muc,M.; Erick,J.; Thomas,P.E.; Kelley,M. Cytochrome P-450 2E1 is not the sole catalyst of chlorzoxazone hydroxylation in rat liver microsomes, *J.Pharmacol.Exp.Ther.*, **1995**, *273*, 1156–1161.

SAMPLE

Matrix: solutions

HPLC VARIABLES

Column: 250 × 4.6 5 μm Supelcosil LC-DP (A) or 250 × 4 5 μm LiChrospher 100 RP-8 (B)
Mobile phase: MeCN:0.025% phosphoric acid:buffer 25:10:5 (A) or 60:25:15 (B) (Buffer was 9 mL concentrated phosphoric acid and 10 mL triethylamine in 900 mL water, adjust pH to 3.4 with dilute phosphoric acid, make up to 1 L.)
Flow rate: 0.6
Injection volume: 25
Detector: UV 229

CHROMATOGRAM

Retention time: 6.01 (A), 5.87 (B)

OTHER SUBSTANCES

Also analyzed: acebutolol, acepromazine, acetaminophen, acetazolamide, acetophenazine, albuterol, alprazolam, amitriptyline, amobarbital, amoxapine, antipyrine, atenolol, atropine, azatadine, baclofen, benzocaine, bromocriptine, brompheniramine, brotizolam, bupivacaine, buspirone, butabarbital, butalbital, caffeine, carbamazepine, cetirizine, chlorcyclizine, chlordiazepoxide, chlormezanone, chloroquine, chlorpheniramine, chlorpromazine, chlorpropamide, chlorprothixene, chlorthalidone, cimetidine, cisapride, clomipramine, clonazepam, clonidine, clozapine, cocaine, codeine, colchicine, cyclizine, cyclobenzaprine, dantrolene, desipramine, diazepam, diclofenac, diflunisal, diltiazem, diphenhydramine, diphenidol, diphenoxylate, dipyridamole, disopyramide, dobutamine,

doxapram, doxepin, droperidol, encainide, ethidium bromide, ethopropazine, fenoprofen, fentanyl, flavoxate, fluoxetine, fluphenazine, flurazepam, flurbiprofen, fluvoxamine, furosemide, glutethimide, glyburide, guaifenesin, haloperidol, homatropine, hydralazine, hydrochlorothiazide, hydrocodone, hydromorphone, hydroxychloroquine, hydroxyzine, ibuprofen, imipramine, indomethacin, ketoconazole, ketoprofen, ketorolac, labetalol, levorphanol, lidocaine, loratadine, lorazepam, lovastatin, loxapine, mazindol, mefenamic acid, meperidine, mephenytoin, mepivacaine, mesoridazine, metaproterenol, methadone, methdilazine, methocarbamol, methotrexate, methotrimeprazine, methoxamine, methyldopa, methylphenidate, metoclopramide, metolazone, metoprolol, metronidazole, midazolam, moclobemide, morphine, nadolol, nalbuphine, naloxone, naphazoline, naproxen, nifedipine, nizatidine, norepinephrine, nortriptyline, oxazepam, oxycodone, oxymetazoline, paroxetine, pemoline, pentazocine, pentobarbital, pentoxifylline, perphenazine, pheniramine, phenobarbital, phenol, phenolphthalein, phentolamine, phenylbutazone, phenyltoloxamine, phenytoin, pimozide, pindolol, piroxicam, pramoxine, prazepam, prazosin, probenecid, procainamide, procaine, prochlorperazine, procyclidine, promazine, promethazine, propafenone, propantheline, propiomazine, propofol, propranolol, protriptyline, quazepam, quinidine, quinine, racemethorphan, ranitidine, remoxipride, risperidone, salicylic acid, scopolamine, secobarbital, sertraline, sotalol, spironolactone, sulfinpyrazone, sulindac, temazepam, terbutaline, terfenadine, tetracaine, theophylline, thiethylperazine, thiopental, thioridazine, thiothixene, timolol, tocainide, tolbutamide, tolmetin, trazodone, triamterene, triazolam, trifluoperazine, triflupromazine, trimeprazine, trimethoprim, trimipramine, verapamil, warfarin, xylometazoline, yohimbine, zopiclone

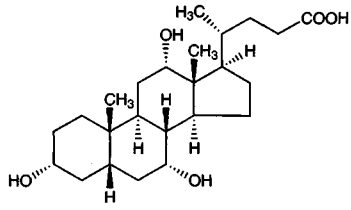
KEY WORDS

also details of plasma extraction

REFERENCE

Koves,E.M. Use of high-performance liquid chromatography-diode array detection in forensic toxicology, *J.Chromatogr.A*, **1995**, *692*, 103–119.

Cholic acid



Molecular formula: $C_{24}H_{40}O_5$
Molecular weight: 408.58
CAS Registry No.: 81-25-4
Merck Index: 2258

SAMPLE

Matrix: bile, blood

Sample preparation: Serum. 100-200 µL Serum + 1 mL MeOH, mix, sonicate for 15 min. Remove a 600 µL aliquot of the supernatant and evaporate it to dryness under a stream of nitrogen, reconstitute with 1 mL 50 mM pH 7.0 phosphate buffer, add to a Sep-Pak C18 SPE cartridge, wash with 2 mL MeOH:water 20:80, elute with 4 mL MeOH:water 80:20. Evaporate the eluate to dryness under reduced pressure at 40°, reconstitute with 1 mL MeOH. Remove a 500 µL aliquot and add it to 50 µL 100 µM lauric acid in MeOH, add 50 µL 0.1 mg/mL KOH on MeOH, evaporate to dryness under a stream of nitrogen, add 100 µL 1 mg/mL dicyclohexyl-18-crown-6 in MeCN, add 100 µL 25 mM 1-bromoacetylpyrene in MeCN, mix, heat at 40° for 30 min, cool, inject an 8 µL aliquot. Bile. Mix 10 µL bile with 10 mL 50 mM pH 7.0 phosphate buffer, add a 1 mL aliquot to a Sep-Pak C18 SPE cartridge, wash with 2 mL MeOH:water 20:80, elute with 4 mL MeOH:water 80:20. Evaporate the eluate to dryness under reduced pressure at 40°, reconstitute with 1 mL MeOH. Remove a 500 µL aliquot and add it to 50 µL 100 µM lauric acid in MeOH, add 50 µL 0.1 mg/mL KOH on MeOH, evaporate to dryness under a stream of nitrogen, add 100 µL 1 mg/mL dicyclohexyl-18-crown-6 in MeCN, add 100 µL 25 mM 1-bromoacetylpyrene in MeCN, mix, heat at 40° for 30 min, cool, inject an 8 µL aliquot.

HPLC VARIABLES

Column: 100 × 8 10 µm Model RCM-100 Radial-Pak A (Waters)
Mobile phase: Gradient. MeCN:MeOH:water 100:50:40 for 30 min then 100:50:20 (step gradient).
Flow rate: 2
Injection volume: 8
Detector: F ex 370 em 440

CHROMATOGRAM

Retention time: 32
Internal standard: lauric acid (56)
Limit of detection: 10 pmole
Limit of quantitation: 50 pmole

OTHER SUBSTANCES

Extracted: chenodiol, deoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid, glycolithocholic acid, glycoursodeoxycholic acid, lithocholic acid, ursodiol

KEY WORDS

derivatization; serum; SPE

REFERENCE

Kamada,S.; Maeda,M.; Tsuji,A. Fluorescence high-performance liquid chromatographic determination of free and conjugated bile acids in serum and bile using 1-bromoacetylpyrene as a pre-labeling reagent, *J.Chromatogr.*, **1983**, *272*, 29–41.

SAMPLE

Matrix: solutions

Sample preparation: Mix an aliquot of solution (or hydrolyzed bile) with a 50% molar excess of triethylamine in MeCN, warm briefly, add a 50% molar excess of 100 mM 2-bromoacetophenone in MeCN, heat at 80-90° for 45-60 min, evaporate to dryness, reconstitute with dioxane (Caution! Dioxane is a carcinogen!), filter (0.47 μm), inject an aliquot.

HPLC VARIABLES
Column: 250 × 4.6 Partisil 10/25 ODS
Mobile phase: Gradient. n-Heptane:dioxane 90:10 for 3 min then n-heptane:dioxane:isopropanol 70:25:5 (step gradient). (Caution! Dioxane is a carcinogen!)
Flow rate: 1.2
Detector: UV 254

CHROMATOGRAM
Retention time: 26
Limit of quantitation: 5 pmole

OTHER SUBSTANCES
Simultaneous: chenodiol, deoxycholic acid, hyodeoxycholic acid, lithocholic acid, ursodiol

KEY WORDS
derivatization

REFERENCE
Stellaard,F.; Hachey,D.L.; Klein,P.D. Separation of bile acids as their phenacyl esters by high-pressure liquid chromatography, *Anal.Biochem.*, **1978**, *87*, 359–366.

SAMPLE
Matrix: solutions
Sample preparation: Treat a solution in MeOH with a slight excess of tetramethylammonium hydroxide in MeOH, evaporate to dryness under a stream of nitrogen, reconstitute with MeCN, add a 2-10 fold excess of 9-(chloromethyl)anthracene in cyclohexane, heat at 75° for 15 min, very dilute solutions may require longer times), dilute with MeCN, inject an aliquot.

HPLC VARIABLES
Column: 300 mm long "Fatty Acid" reversed-phase (Waters)
Mobile phase: MeOH:water 88:12 (A) or 82:18 (B)
Flow rate: 0.75
Detector: UV 254

CHROMATOGRAM
Retention time: 20

OTHER SUBSTANCES
Simultaneous: chenodiol, deoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid

KEY WORDS
derivatization

REFERENCE
Korte,W.D. 9-(Chloromethyl)anthracene: a useful derivatizing reagent for enhanced ultraviolet and fluorescence detection of carboxylic acids with liquid chromatography, *J.Chromatogr.*, **1982**, *243*, 153–157.

SAMPLE
Matrix: solutions

Sample preparation: Mix 200 μL of a solution of bile acids with 50 μL 2.1 mg/mL 2-bromoacetyl-6-methoxynaphthalene in acetone, add 300 μL 10 mM tetrakis(decyl)ammonium bromide in 100 mM pH 7.0 phosphate buffer, heat at 40° for with sonication 10 min, add 300 μL 5.1 μM IS in MeCN, sonicate at room temperature for 1 min, inject a 50 μL aliquot. (Prepare 2-bromoacetyl-6-methoxynaphthalene by stirring equimolar amounts of 2-acetyl-6-methoxynaphthalene (Janssen Chimica, Belgium) and phenyltrimethylammonium tribromide in THF at room temperature for 3 h (Phosphorus and Sulfur 1985, 25, 357), purify by column chromatography on silica gel with chloroform:petroleum ether 50:50 (mp 109-112°) (Chromatographia 1992, 33, 13).)

HPLC VARIABLES

Column: 250 × 4.6 Ultracarb 5 ODS
Mobile phase: Gradient. A was water. B was MeCN:MeOH 60:40. A:B 55:45 for 20 min, to 30:70 over 10 min, maintain at 30:70 for 25 min, return to initial conditions over 5 min.
Column temperature: 35
Flow rate: 1.2
Injection volume: 50
Detector: F ex 300 em 460

CHROMATOGRAM

Retention time: 16
Internal standard: 6-methoxynaphthacyl ester of lauric acid (36)
Limit of detection: 1-2 pmole

OTHER SUBSTANCES

Simultaneous: chenodiol, deoxycholic acid, lithocholic acid, ursodiol

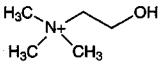
KEY WORDS

derivatization

REFERENCE

Gatti,R.; Roda,A.; Cerre,C.; Bonazzi,D.; Cavrini,V. HPLC-fluorescence determination of individual free and conjugated bile acids in human serum, *Biomed.Chromatogr.*, **1997**, *11*, 11–15.

Choline



Molecular formula: $C_5H_{14}NO$

Molecular weight: 107.17

CAS Registry No.: 62-49-7, 67-48-1 (chloride), 4201-78-9 (dehydrocholate), 77-91-8 (dihydrogen citrate), 2016-36-6 (salicylate), 4499-40-5 (theophyllinate), 87-67-2 (bitartrate), 507-30-2 (gluconate)

Merck Index: 2259

SAMPLE

Matrix: CSF, dialysate, tissue

Sample preparation: Dialysate. Inject dialysate directly. Tissue. Homogenize brain tissue with 10 volumes 100 mM perchloric acid (Potter-Elvehjem), let stand on ice for 15 min, centrifuge at 4000 g for 15 min, inject a 0.5 μL aliquot. CSF. Deproteinize by passing through a 0.02 μm Anatop 10 syringe filter (Alltech), inject a 0.5 μL aliquot of the filtrate.

HPLC VARIABLES

Column: 100 × 1 PEEK column packed with Aminex A-9 (Bio-Rad)

Mobile phase: 200 mM pH 8.0 K/Na 3/1 phosphate buffer containing 5 mM NaCl and 0.1% Kathon CG

Column temperature: 25

Flow rate: 0.06 (obtained with a flow splitter)

Injection volume: 0.5

Detector: E, AMOR (Spark Holland), platinum working electrode + 250 mV, carbon composite auxiliary electrode, Ag/AgCl reference electrode, following post-column reaction. The column effluent flowed through a reactor which had 4 U acetylcholine esterase (EC 3.1.1.7 type VI-S from electric eel, 260 IU/mg) and 4 U choline oxidase (EC 1.1.3.17 from Alcaligenes sp., 12.7 IU/mg) enclosed between two 0.01 μm cellulose nitrate filters (Sartorius) (construction details given) to the detector.

CHROMATOGRAM

Retention time: 8

Limit of detection: 10 fmole

OTHER SUBSTANCES

Extracted: acetylcholine

KEY WORDS

use metal-free tubing and connectors; solvent reservoir; column; reactor; and detector maintained at 25; rat; brain; human; post-column reaction; immobilized enzyme reactor

REFERENCE

Flentge,F.; Venema,K.; Koch,T.; Korf,J. An enzyme-reactor for electrochemical monitoring of choline and acetylcholine: applications in high-performance liquid chromatography, brain tissue, microdialysis and cerebrospinal fluid, *Anal.Biochem.*, **1992**, *204*, 305–310.

SAMPLE

Matrix: blood

Sample preparation: 250 μL Plasma + 25 μL 0.41 mM IS + 750 μL ice-cold 1 M formic acid in acetone, mix, centrifuge for 15 min. Remove the supernatant and add it to the SPE column, wash with 1 mL 100 mM pH 4.0 ammonium acetate, elute with 1 mL 2 M NaCl in MeOH:water 50:50, force out all liquid under pressure. Evaporate the eluate under a stream of nitrogen and keep under vacuum for 1 h, add 1 mL MeCN to the residue, mix, centrifuge, remove the supernatant, repeat the extraction. Combine the supernatants and evaporate them to dryness under a stream of nitrogen, reconstitute the residue in 300 μL freshly prepared 21.7 mM 3,5-dinitrobenzoyl chloride in pyridine (dry

pyridine over KOH), heat at 105° for 1 h, evaporate the pyridine under a stream of nitrogen, extract the residue with 300 μL water then with 200 μL water, combine the extracts, filter (0.3 μm), inject a 100 μL aliquot. (Preparation of SPE column. Let 300 mg AG 50W-X12 cation-exchange resin sit overnight in 1 mL 100 mM pH 4.0 ammonium acetate. Add the mixture to a Pasteur pipette with a glass wool plug, wash column with 1 mL 2 M NaCl in MeOH:water 50:50, activate column with 1 mL 100 mM pH 4.0 ammonium acetate.)

HPLC VARIABLES

Column: Two 300 × 4 10 μm μBondapak C18 in series
Mobile phase: MeCN:water 50:50 containing 5 mM sodium dodecyl sulfate and 0.1% acetic acid
Flow rate: 2-2.3
Injection volume: 100
Detector: UV 254

CHROMATOGRAM

Retention time: 13
Internal standard: 3-hydroxy-N,N,N-trimethylpropanaminium iodide (15) (Prepare by adding 18.5 g iodomethane to 10 g 3-hydroxy-N,N-dimethylaminopropane in 24 mL EtOH, stir, filter, add cold diethyl ether to the filtrate, filter. Combine precipitates, recrystallize from EtOH/diethyl ether, mp 203-4°)
Limit of quantitation: 1 nmole/mL

KEY WORDS

plasma; SPE; derivatization

REFERENCE

Buchanan,D.N.; Fucek,F.R.; Domino,E.F. Paired-ion high-performance liquid chromatographic assay for plasma choline, *J.Chromatogr.*, **1980**, *181*, 329–335.

SAMPLE

Matrix: blood
Sample preparation: Add neostigmine and ethylhomocholine to plasma or red blood cells. 150 μL Plasma or red blood cells + 1 mL 400 mM perchloric acid, let stand at 0-4° for 30 min, centrifuge at 5500 g for 1 min. Remove a 750 μL aliquot of the supernatant and add it to 36 μL 10 M potassium acetate, let stand at 0-4° for 5 min, centrifuge at 5500 g for 1 min, inject a 100 μL aliquot.

HPLC VARIABLES

Guard column: 10 × 1.9 Chrompack reversed phase
Column: 100 × 3 Chromspher 5 C18 (Chrompack) (Prepare column by washing with MeOH, MeOH:water 50:50, water, and 5 mg/mL sodium laurylsulfate in water (each wash 20 min at 1 mL/min). Thoroughly wash pump with water (column off line), wash column with water for 5 min and mobile phase for 1 h. Column should be disconnected from the precolumn, reactor, and detector. [Chromatographia, 1987,24,827].)
Mobile phase: 200 mM pH 8.0 potassium phosphate buffer containing 5 mM KCl
Flow rate: 0.6
Injection volume: 100
Detector: E, Spark Holland Amor, Pt working electrode +500 mV, carbon composite auxiliary electrode, Ag/AgCl reference electrode following an enzyme reactor. (Reactor was a 10 × 2.1 Hypersil APS-2 column, activate with glutaraldehyde, equilibrate with mobile phase for 20 min. Inject 80 U acetylcholine esterase (EC 3.1.1.7, type VI-S from electric eel, 260 IU/mg) and 40 U choline oxidase (EC 1.1.3.17 from Alcaligenes sp., 12.7 IU/mg) in 500 μL mobile phase onto the reactor and pump through at 0.05 mL/min for 20 min with mobile phase. [Chromatographia, 1987,24,827])

CHROMATOGRAM
Retention time: 2.5
Internal standard: ethylhomocholine (5)
Limit of detection: 10 nM

OTHER SUBSTANCES
Extracted: acetylcholine

KEY WORDS
plasma; red blood cells; human; mouse; post-column reaction; immobilized enzyme reactor

REFERENCE
Damsma,G.; Flentge,F. Liquid chromatography with electrochemical detection for the determination of choline and acetylcholine in plasma and red blood cells. Failure to detect acetylcholine in blood of humans and mice, *J.Chromatogr.*, **1988**, *428*, 1–8.

SAMPLE
Matrix: blood
Sample preparation: 100 μL Plasma + 2 mL 300 ng/mL ethylhomocholine in MeCN, vortex for 1 min, centrifuge at 2000 g for 15 min. Remove 1.5 mL of the supernatant and evaporate it to dryness under vacuum, reconstitute the residue in 500 μL mobile phase, inject a 50 μL aliquot.

HPLC VARIABLES
Column: 100 × 4.6 5 μm Cyano Spheri-5
Mobile phase: 20 mM sodium hydrogen phosphate and 10 mM tetramethylammonium chloride adjusted to pH 7.1 with 65% phosphoric acid
Flow rate: 0.7
Injection volume: 50
Detector: E, ESA Coulochem II, Model 5040 Pt analytical cell + 300 mV, following an enzyme reactor. (The reactor was a 30 × 2.1 7 μm Aquapore AX-300 anion-exchange cartridge (Brownlee), inject slowly 50 μL 100 U/mL choline oxidase (EC 1.1.3.17, Alcaligenes) and catalase (EC 1.11.1.6) (Sigma), wash with mobile phase for several minutes before use, reload after 100 samples.)

CHROMATOGRAM
Retention time: 5
Internal standard: ethylhomocholine (8)
Limit of quantitation: 3.58 μM

KEY WORDS
pharmacokinetics; immobilized enzyme reactor; plasma; post-column reaction

REFERENCE
Fossati,T.; Colombo,M.; Castiglioni,C.; Abbiati,G. Determination of plasma choline by high-performance liquid chromatography with a postcolumn enzyme reactor and electrochemical detection, *J.Chromatogr.B*, **1994**, *656*, 59–64.

SAMPLE
Matrix: bulk
Sample preparation: Heat 500 nmole with 500 μL 1 mg/mL benoxaprofen chloride in MeCN or toluene at 60-80° for 1 h, inject a 10 μL aliquot. (Prepare benoxaprofen chloride as follows. Dissolve 600 mg benoxaprofen in 50 mL dry toluene, slowly add 5 mL thionyl chloride (freshly distilled from linseed oil), reflux for 30 min, evaporate to dryness, recrystallize from dichloromethane (if necessary) to give benoxaprofen chloride (mp 91.5°).)

HPLC VARIABLES
Column: 120 × 4.6 5 μm LiChrosorb RP-8

Mobile phase: Acetone:10 mM sodium heptanesulfonate:phosphoric acid 60:40:0.15
Column temperature: 55
Flow rate: 2
Injection volume: 10
Detector: F ex 312 em 365

CHROMATOGRAM
Retention time: 7.5

OTHER SUBSTANCES
Simultaneous: scopolamine N-butylbromide

KEY WORDS
derivatization

REFERENCE
Spahn,H.; Weber,H.; Mutschler,E.; Möhrke,W. α-Alkyl-α-arylacetic acid derivatives as fluorescence markers for thin-layer chromatographic and high-performance liquid chromatographic assay of amines and alcohols, *J.Chromatogr.*, **1984**, *310*, 167–178.

SAMPLE
Matrix: bulk, formulations
Sample preparation: Dilute succinylcholine chloride bulk drug and formulation samples in MeCN:100 mM hexanesulfonic acid:water 5:20:75, inject an aliquot. (Prepare the diluent by diluting 100 mL 100 mM hexanesulfonic acid and 25 mL MeCN to 500 mL with water.)

HPLC VARIABLES
Column: 250 × 4.6 5 μm Alltima C18
Mobile phase: Gradient. A was MeCN:5 mM hexanesulfonic acid:water 5:5:90. B was MeCN:water 50:50. A:B 100:0 for 13 min, from 100:0 to 0:100 in 2 min, maintain at 0:100 for 10 min, from 0:100 to 100:0 in 2 min, maintain at 100:0 for 8 min
Flow rate: 1
Injection volume: 50
Detector: Conductivity, Waters M-430 conductivity detector coupled with a Dionex CMMS-II cation micromembrane suppressor with suppression regenerant of 25 mM tetrabutylammonium hydroxide at 5 mL/min

CHROMATOGRAM
Retention time: 11
Limit of detection: 10 pmol

OTHER SUBSTANCES
Noninterfering: succinylcholine chloride

REFERENCE
Chen,S.; Soneji,V.; Webster,J. Determination of choline in pharmaceutical formulations by reversed-phase high-performance liquid chromatography and postcolumn suppression conductivity detection, *J.Chromatogr.A*, **1996**, *739*, 351–357.

SAMPLE
Matrix: dialysate
Sample preparation: Inject 10 μL of rat brain dialysate.

HPLC VARIABLES
Guard column: ACH-3-G guard cartridge (ESA)
Column: 150 × 3 5 μm ACH-3 polymeric reversed-phase column (ESA)

Mobile phase: 100 mM sodium phosphate + 0.5 mM tetramethylammonium chloride + 0.005% Reagent MB (a microbicide from ESA) + 2 mM octanesulfonic acid, final pH 8.0
Column temperature: 35
Flow rate: 0.35
Injection volume: 10
Detector: E, ESA Coulochem Model 5200A, Model 5040 analytical cell, palladium reference electrode, stainless steel counter electrode, platinum working electrode + 300 mV following a solid-phase reactor containing immobilized acetylcholinesterase and choline oxidase (reactor temp 35)

CHROMATOGRAM
Retention time: 4
Limit of detection: 2 μM

OTHER SUBSTANCES
Extracted: acetylcholine

KEY WORDS
brain; rat; post-column reaction; immobilized enzyme reactor

REFERENCE
Greaney,M.D.; Marshall,D.L.; Bailey,B.A.; Acworth,I.N. Improved method for the routine analysis of acetylcholine release in vivo: quantitation in the presence and absence of esterase inhibitor, *J.Chromatogr.*, **1993**, *622*, 125–135.

SAMPLE
Matrix: dialysate
Sample preparation: Inject 5 μL aliquots of the dialysate (Ringer's solution).

HPLC VARIABLES
Column: 530 × 1 cation exchange MF-8904 (Bioanalytical Systems)
Mobile phase: 50 mM Na_2HPO_4 containing 5 mL/L Kathon CG (Bioanalytical Systems CF-2150) (Mobile phase was only partially degassed; some oxygen is essential for the enzyme reactor.)
Flow rate: 0.14
Injection volume: 5
Detector: E, Bioanalytical Systems LC-4C, peroxidase-redox polymer coated glassy carbon electrode +100 mV (Anal.Chem. 1992, 64, 3084), Ag/AgCl reference electrode. The column effluent passed through a 50 × 1 immobilized-enzyme reactor containing acetylcholinesterase (EC 3.1.1.7) and choline oxidase (EC 1.1.3.17) (Bioanalytical Systems MF-8903) and flowed to the detector.

CHROMATOGRAM
Retention time: 24
Limit of detection: 10 fmole

OTHER SUBSTANCES
Extracted: acetylcholine

KEY WORDS
rat; post-column reaction; immobilized enzyme reactor

REFERENCE
Huang,T.; Yang,L.; Gitzen,J.; Kissinger,P.T.; Vreeke,M.; Heller,A. Detection of basal acetylcholine in rat brain microdialysate, *J.Chromatogr.B*, **1995**, *670*, 323–327.

SAMPLE
Matrix: formulations

Sample preparation: Make up the lyophilized preparation in sterile water, inject a 50 μL aliquot.

HPLC VARIABLES
Column: μBondapak C18
Mobile phase: Mix (? g) sodium 1-heptanesulfonate (Waters PIC Reagent B-7) in 900 mL water, adjust pH to 4.0 with 6 M ammonium hydroxide, add 50 mL MeCN, make up to 1 L with water
Flow rate: 2
Injection volume: 50
Detector: RI

CHROMATOGRAM
Retention time: 5

OTHER SUBSTANCES
Simultaneous: acetylcholine
Noninterfering: acetic acid, mannitol

REFERENCE
Tao,F.T.; Thurber,J.S.; Dye,D.M. High-performance liquid chromatographic determination of acetylcholine in a pharmaceutical preparation, *J.Pharm.Sci.*, **1984**, *73*, 1311–1313.

SAMPLE
Matrix: formulations
Sample preparation: Inject a 10 μL aliquot.

HPLC VARIABLES
Column: 250 × 0.26 silica A (Perkin-Elmer)
Mobile phase: MeOH:water:500 mM tetramethylammonium sulfate 65:25:10
Flow rate: 1
Injection volume: 10
Detector: UV 214

CHROMATOGRAM
Retention time: 2.9

OTHER SUBSTANCES
Simultaneous: methyl p-hydroxybenzoate, succinic acid, succinylcholine, succinylmonocholine

KEY WORDS
injections; saline

REFERENCE
Schmutz,C.W.; Mühlebach,S.F. Stability of succinylcholine chloride injection, *Am.J.Hosp.Pharm.*, **1991**, *48*, 501–506.

SAMPLE
Matrix: formulations
Sample preparation: Condition a Sep Pak C18 SPE cartridge with 2 mL MeOH and 2 mL water, pass the formulation through the SPE cartridge to remove fat-soluble vitamins, inject an aliquot of the eluate.

HPLC VARIABLES
Column: 125 × 4 5 μm Nucleosil SA or 300 × 4.6 5 μm Zorbax SCX 300 Å

Mobile phase: EtOH:100 mM pH 5.0 ammonium acetate 20:80 containing 0.3 mM 3-hydroxytyramine hydrochloride (After use wash column with EtOH:water 20:80. Keep column in EtOH:water 70:30 when not in use.)
Column temperature: 40
Flow rate: 1.2
Injection volume: 20
Detector: UV 280

CHROMATOGRAM
Retention time: 5.3

KEY WORDS
indirect UV detection; SPE

REFERENCE
Leroy,P.; Barbaras,M.; Colin,J.L.; Nicolas,A. Ion-exchange liquid chromatography method with indirect UV detection for the assay of choline in pharmaceutical preparations, *J.Pharm.Biomed.Anal.*, **1995**, *13*, 581–588.

SAMPLE
Matrix: solutions
Sample preparation: Prepare a 4 mM solution in water, inject a 10 μL aliquot

HPLC VARIABLES
Column: μBondapak C18 Radial-Pak in a RCM-100 radial compression module
Mobile phase: Butanol:MeOH:acetic acid:water 8:4:2:86 containing 0.15 mM 1-phenethyl-2-picolinium bromide (Extract 10 mM 1-phenethyl-2-picolinium bromide stock solution with dichloromethane before use to remove UV-absorbing impurities.)
Flow rate: 3
Injection volume: 10
Detector: UV 254

CHROMATOGRAM
Retention time: 2

OTHER SUBSTANCES
Simultaneous: acetylcholine, butyrylcholine, propionylcholine

REFERENCE
Jones,R.S.; Stutte,C.A. Chromatographic analysis of choline and acetylcholine by UV visualization, *J.Chromatogr.*, **1985**, *319*, 454–460.

SAMPLE
Matrix: solutions
Sample preparation: Prepare a solution in n-propanol:water 80:20 or DMF:water 80:20, inject an aliquot.

HPLC VARIABLES
Column: 250 × 4 5 μm LiChrospher 100 Diol
Mobile phase: Gradient. A was hexane. B was ethyl acetate. C was 0.1% formic acid in MeCN. D was 0.1% formic acid in water. A:B:C:D 100:0:0:0 for 5 min, to 0:100:0:0 over 15 min, maintain at 0:100:0:0 for 5 min, to 0:0:100:0 over 5 min, maintain at 0:0:100:0 for 5 min; to 0:0:0:100 over 25 min, maintain at 0:0:0:100 for 5 min.
Flow rate: 0.9
Detector: Evaporative light scattering (Sédex 55, Sédéré)

CHROMATOGRAM
Retention time: 55.04

OTHER SUBSTANCES

Simultaneous: acetylcholine, cholesterol, cortisone, dextrose, estradiol, glycine, phenylalanine, sodium, testosterone

REFERENCE

Treiber,L.R. Normal-phase high-performance liquid chromatography with relay gradient elution. I. Description of the method, *J.Chromatogr.A*, **1995**, *696*, 193–199.

SAMPLE

Matrix: solutions

Sample preparation: Prepare a solution in n-propanol:water 80:20 or DMF:water 80:20, inject an aliquot.

HPLC VARIABLES

Column: 250 × 4 5 μm LiChrospher 100 Diol

Mobile phase: Gradient. A was 0.1% trifluoroacetic acid in MeCN. B was 0.1% trifluoroacetic acid in water. A:B 90:10 for 1 min, to 70:30 over 17 min, to 0:100 over 2 min, maintain at 0:100 over 4.5 min

Flow rate: 0.9

Detector: Evaporative light scattering (Sédex 55, Sédéré)

CHROMATOGRAM

Retention time: 5.08

OTHER SUBSTANCES

Simultaneous: acetylcholine, sodium, magnesium, calcium

REFERENCE

Treiber,L.R. Normal-phase high-performance liquid chromatography with relay gradient elution. I. Description of the method, *J.Chromatogr.A*, **1995**, *696*, 193–199.

SAMPLE

Matrix: tissue

Sample preparation: Homogenize brain tissue with 3 mL 400 mM perchloric acid containing 2 nmoles ethylhomocholine, centrifuge at 35000 g for 20 min, adjust pH of supernatant to about 4.2 with about 200 μL 7.5 M potassium acetate, centrifuge at 35000 g for 20 min. Add the supernatant to 100 μL 5 mM tetramethylammonium chloride, add 3 mL 2% ice-cold reineckate solution, let stand on ice for 1 h, centrifuge at 1000 g at 0° for 10 min. Remove the supernatant and dry the precipitate under vacuum overnight, add about 1 mL 5 mM silver tosylate in MeCN until the pink color disappears, centrifuge at 1000 g at 0° for 10 min. Remove the supernatant and evaporate it to dryness under a stream of nitrogen, reconstitute the residue in 200 μL 20 mM pH 3.5 citrate-phosphate buffer, inject an aliquot.

HPLC VARIABLES

Guard column: ODS-5 (Bio-Rad)

Column: 150 mm long Bio-Sil ODS-5S (Bio-Rad)

Mobile phase: Buffer (Buffer was 10 mM sodium acetate buffered to pH 5 with 20 mM citric acid, containing 4.5 mg/L sodium octyl sulfate and 1.2 mM tetramethylammonium chloride.)

Flow rate: 0.8

Injection volume: 20

Detector: E, Bio Analytical Systems LC-4A, Pt electrode +0.5 V, Ag/AgCl reference electrode following post-column reaction detection. The column effluent mixed with 1 U/mL choline oxidase and 2 U/mL acetylcholinesterase in 200 mM pH 8.5 phosphate buffer pumped at 0.5 mL/min, the mixture flowed through a 30 m × 0.3 mm i.d. PTFE tube (2.5 min) to the detector

CHROMATOGRAM
Retention time: 3.6
Internal standard: ethylhomocholine (7.2)
Limit of detection: 1 pmole

OTHER SUBSTANCES
Extracted: acetylcholine

KEY WORDS
post-column reaction; rat; brain; immobilized enzyme reactor

REFERENCE
Potter,P.E.; Meek,J.L.; Neff,N.H. Acetylcholine and choline in neuronal tissue measured by HPLC with electrochemical detection, *J.Neurochem.*, **1983**, *41*, 188–194.

SAMPLE
Matrix: tissue
Sample preparation: Sonicate 250 mg rat brain tissue with 6 mL 1 M formic acid containing 10 nmoles IS for 5 min, centrifuge at 4° at 10000 g for 20 min, add the supernatant to an equal volume of diethyl ether, add 5 mL water, shake, centrifuge at 2000 g for 5 min, discard the organic layer. Lyophilize the aqueous layer, dissolve the residue in 400 μL water, filter (0.45 μm). Add 30 μL reagent to the filtrate, mix, centrifuge at 10000 g for 5 min. Dissolve the precipitate in 300 μL water, add 50 mg Dowex 1x8, shake, centrifuge at 10000 g for 5 min, inject a 10 μL aliquot of the supernatant. (Reagent contained 20% KI and 18% iodine in water.)

HPLC VARIABLES
Column: 150 × 4.6 Nucleosil C18
Mobile phase: Buffer (Prepare buffer by dissolving 1.36 g sodium acetate. 3.72 disodium EDTA, 25 mg sodium octyl sulfate, and 1.2 mmoles tetramethylammonium chloride in 900 mL water, adjust pH to 5.0 with 200 mM citric acid, make up to 1 L.)
Column temperature: 37
Flow rate: 0.8
Injection volume: 10
Detector: E, Bioanalytical Systems LC-4B/17, TL-10A platinum electrode +500 mV, Ag/AgCl reference electrode following post-column reaction. The effluent from the column mixed with buffer pumped at 0.5 mL/min and the mixture flowed through an immobilized enzyme reactor to the detector. (Prepare buffer by dissolving 71.6 g $Na_2HPO_4.12H_2O$ and 372 mg disodium EDTA in 900 mL water, adjust pH to 8.5 with NaH_2PO_4, make up to 1 L. Prepare reactor by heating 200-400 mesh porous glass beads (pore size 400 Å, Electronucleonics, Fairfield NJ) in 5% nitric acid, wash with water, dry, add to 10% 3-aminopropyltriethoxysilane in toluene, reflux overnight. Suspend the beads in 2% glutaraldehyde in water at room temperature for 2 h. Dissolve 0.45 mg acetylcholinesterase (type III, EC.3.1.1.7, Sigma) and 16.6 mg choline oxidase (EC.1.1.3.17, Sigma) in 200 μL 50 mM pH 7.0 phosphate buffer containing 10 mM disodium EDTA, add 500 mg activated beads, pack in a 10 × 4 tube.)

CHROMATOGRAM
Retention time: 6
Internal standard: ethylhomocholine (Prepare ethylhomocholine by adding 3-dimethylamino-1-propanol to EtOH, add bromoethane. When reaction is complete add ether, filter off the precipitate and wash it with ether.) (8)
Limit of detection: 100 fmoles

OTHER SUBSTANCES
Extracted: acetylcholine

KEY WORDS
post-column reaction; rat; brain; immobilized enzyme reactor

REFERENCE
Asano,M.; Miyauchi,T.; Kato,T.; Fujimori,K.; Yamamoto,K. Determination of acetylcholine and choline in rat brain tissue by liquid chromatography/electrochemistry using an immobilized enzyme post column reactor, *J.Liq.Chromatogr.*, **1986**, *9*, 199–215.

SAMPLE
Matrix: tissue
Sample preparation: Sonicate rat brain with ten volumes of 1 M formic acid:acetone 15:85 containing IS, centrifuge at 4° at 20000 g. Remove a 500 μL aliquot of the supernatant and add it to 2 mL heptane:chloroform 80:10, vortex. Remove the aqueous layer and add it to 250 μL 3 mg/mL sodium tetraphenylboron in 3-heptanone, vortex. Remove a 200 μL aliquot of the upper organic layer and add it to 50 μL 1 M HCl, vortex. Remove the aqueous layer and evaporate it to dryness under reduced pressure, reconstitute with mobile phase, inject a 30 μL aliquot.

HPLC VARIABLES
Guard column: C18 (Waters)
Column: 250 × 4.6 5 μm Hypersil ODS
Mobile phase: 100 mM pH 7 KH_2PO_4 containing 10 μg/mL sodium octane sulfate and 600 μg/mL tetramethylammonium chloride
Flow rate: 1
Injection volume: 30
Detector: E, Chromatofield, Pt electrode +0.5 V following post-column reaction. The column effluent flowed through an immobilized enzyme reactor to the detector. (Prepare reactor by heating 200-400 mesh porous glass beads (pore size 350 Å, Sigma) in 5% nitric acid at 100° for 1 min, wash with water, dry, add to 10% 3-aminopropyltriethoxysilane in toluene, heat at 115° for 12 h. Suspend the beads in 2% glutaraldehyde in water at room temperature for 2 h. Dissolve 100 U acetylcholinesterase (type III, electric eel, Sigma) and 100 U choline oxidase (Alcaligenes, Sigma) in 1 mL 50 mM pH 7 phosphate buffer, add 120 mg activated beads, shake periodically, pack in a 20 × 2 tube.)

CHROMATOGRAM
Retention time: 4.5
Internal standard: ethylhomocholine bromide (Prepare ethylhomocholine by adding 3-dimethylamino-1-propanol to EtOH, add bromoethane, when reaction is complete add ether, filter off the precipitate and wash it with ether.) (7)

OTHER SUBSTANCES
Extracted: acetylcholine

KEY WORDS
rat; brain; post-column reaction; immobilized enzyme reactor

REFERENCE
Beley,A.; Zekhnini,A.; Lartillot,S.; Fage,D.; Bralet,J. Improved method for determination of acetylcholine, choline, and other biogenic amines in a single brain tissue sample using high performance liquid chromatography and electrochemical detection, *J.Liq.Chromatogr.*, **1987**, *10*, 2977–2992.

SAMPLE
Matrix: tissue
Sample preparation: Homogenize brain tissue with 1 mL 50 mM perchloric acid containing 10 nmoles ethylhomocholine for 1 min (Nissei Model US-300T, 300 W, 20 kHz), centrifuge at 20000 g at 4° for 15 min, filter (0.45 μm), inject a 10 μL aliquot of the filtrate.

HPLC VARIABLES

Guard column: 10 × 4 glassy carbon particles IRICA Type CP-2250 (IRICA Instruments) (removes interfering catecholamines but is not essential)
Column: 60 × 4 3 μm Acetylcholine Separation polymeric styrene-based column (Bioanalytical Systems)
Mobile phase: 50 mM pH 8.4 phosphate containing 1 mM disodium EDTA and 0.4 mM sodium 1-octanesulfonate
Column temperature: 35
Flow rate: 0.8
Injection volume: 20
Detector: E, Bioanalytical systems LC-4A, dual platinum electrodes + 0.50 V, Ag/AgCl reference electrode, following a 5 × 4 reactor containing immobilized acetylcholinesterase and choline oxidase

CHROMATOGRAM

Retention time: 2.04
Internal standard: ethylhomocholine (4.07)

OTHER SUBSTANCES

Extracted: acetylcholine

KEY WORDS

rat; brain; post-column reaction; immobilized enzyme reactor

REFERENCE

Ikarashi,Y.; Iwatsuki,H.; Blank,C.L.; Maruyama,Y. Glassy carbon pre-column for direct determination of acetylcholine and choline in biological samples using liquid chromatography with electrochemical detection, *J.Chromatogr.*, **1992**, *575*, 29–37.

SAMPLE

Matrix: tissue
Sample preparation: Tissue. Homogenize brain tissue with 10 volumes 400 mM perchloric acid, centrifuge. Remove the supernatant and add it to one tenth the volume of 7.5 M potassium acetate solution, centrifuge. Remove a 100 μL aliquot and take it to dryness in a vacuum centrifuge, dissolve the residue in mobile phase, inject an aliquot. CSF, plasma. Add two volumes of 96% EtOH to CSF or plasma, centrifuge. Remove a 100 μL aliquot and take it to dryness in a vacuum centrifuge, dissolve the residue in mobile phase, inject an aliquot.

HPLC VARIABLES

Column: 60 × 4.6 5 μm Bakerbond Sulfopropyl
Mobile phase: 100 mM pH 7.5 sodium phosphate with 5 mM tetramethylammonium chloride
Flow rate: 1.2
Detector: E, Biometra EP 20, platinum electrode + 0.5 V following an immobilized enzyme reactor containing choline oxidase (EC 1.1.3.17) and acetylcholine esterase (EC 3.1.1.7) to convert acetylcholine and choline to hydrogen peroxide which was then detected

CHROMATOGRAM

Retention time: 2.1
Limit of detection: 0.1 pmol

OTHER SUBSTANCES

Extracted: acetylcholine

KEY WORDS

rat; brain; post-column reaction; immobilized enzyme reactor

REFERENCE

Klein,J.; Gonzalez,R.; Köppen,A.; Löffelholz,K. Free choline and choline metabolites in rat brain and body fluids: sensitive determination and implications for choline supply to the brain, *Neurochem.Int.*, **1993**, *22*, 293–300.

SAMPLE

Matrix: tissue

Sample preparation: Homogenize (Nissei US-300T ultrasonic cell disrupter at 300 W and 20 kHz for 1 min) rat brain striatal tissue with 1 mL 1 μM ethylhomocholine in 50 mM perchloric acid, centrifuge at 4° at 20000 g for 15 min, filter (0.45 μm) the supernatant, inject a 10 μL aliquot.

HPLC VARIABLES

Guard column: 10 × 4 74-149 μm plastic formed carbon (details in paper)

Column: 60 × 4 3 μm Acetylcholine Separation polymeric styrene column (Bioanalytical Systems)

Mobile phase: 50 mM pH 8.40 Phosphate buffer containing 1 mM disodium EDTA and 0.40 mM sodium 1-octanesulfonate

Column temperature: 35 ± 1

Flow rate: 0.7

Injection volume: 10

Detector: E, Bioanalytical Systems LC-4A, dual Pt working electrode +500 mV, Ag/AgCl reference electrode following post-column reaction. The effluent from the column flowed through a 5 × 4 immobilized enzyme reactor containing acetylcholinesterase and choline oxidase (Bioanalytical Systems) to the detector.

CHROMATOGRAM

Retention time: 3.5

Internal standard: ethylhomocholine (6.5)

OTHER SUBSTANCES

Extracted: acetylcholine

Noninterfering: 3,4-dihydroxybenzylamine, dopamine, norepinephrine

KEY WORDS

rat; brain; guard-column removes interferences from catecholamines; post-column reaction; immobilized enzyme reactor

REFERENCE

Ikarashi,Y.; Blank,C.L.; Suda,Y.; Kawakubo,T.; Maruyama,Y. Application of a novel, plastic formed carbon as a precolumn packing material for the liquid chromatographic determination of acetylcholine and choline in biological samples, *J.Chromatogr.A*, **1995**, *718*, 267–272.

Chymopapain

Molecular formula: indefinite
CAS Registry No.: 9001-09-6
Merck Index: 2319

SAMPLE
Matrix: solutions

HPLC VARIABLES
Column: 75 × 7.5 TSK SP 5PW (Toyo Soda)
Mobile phase: Gradient. 20 mM pH 5.0 sodium acetate buffer:100 mM pH 5.0 sodium acetate buffer 70:30 to 0:100 over 75 min
Flow rate: 1
Detector: UV 280

CHROMATOGRAM
Retention time: 19, 44 (most active fractions)

KEY WORDS
commercial chymopapain is a complex mixture

REFERENCE
Calam,D.H.; Davidson,J.; Harris,R. High-performance liquid chromatographic investigations on some enzymes of papaya latex, *J.Chromatogr.*, **1985**, *326*, 103–111.

Chymotrypsin

Molecular formula: indefinite
CAS Registry No.: 9004-07-3
Merck Index: 2320

SAMPLE

Matrix: solutions

HPLC VARIABLES

Column: 250 × 2.6 ODS-HC/Sil-X-1 (Perkin-Elmer)
Mobile phase: Gradient. MeCN containing 0.07% trifluoroacetic acid:0.1% trifluoroacetic acid in water from 0:100 to 60:40 over 30 min
Flow rate: 2
Detector: UV 206

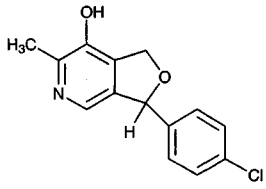
CHROMATOGRAM

Retention time: 25

REFERENCE

Titani,K.; Sasagawa,T.; Resing,K.; Walsh,K.A. A simple and rapid purification of commercial trypsin and chymotrypsin by reverse-phase high-performance liquid chromatography, *Anal.Biochem.*, **1982**, *123*, 408–412.

Cicletanine



Molecular formula: $C_{14}H_{12}ClNO_2$
Molecular weight: 261.71
CAS Registry No.: 89943-82-8, 82747-56-6 (HCl)
Merck Index: 2323
Lednicer No.: 5 143

SAMPLE

Matrix: blood
Sample preparation: 100 μL Plasma + 200 μL 500 ng/mL IS in 250 mM pH 2.5 citrate buffer, inject a 50 μL aliquot onto column A with mobile phase A, after 1 min backflush the contents of column A onto column B with mobile phase B, after 2 min remove column A from the circuit, elute column B with mobile phase B, monitor the effluent from column B, re-equilibrate column A with mobile phase A.

HPLC VARIABLES

Column: A 50 × 2 37-50 μm Bondapak Cx/Corasil (ion-exchange); B 23 × 3.9 37-50 μm Bondapak C18/Corasil + 100 × 8 4 μm Nova-Pak C18 Radial Pak
Mobile phase: A water; B MeCN:100 mM pH 2.5 KH_2PO_4 25:75
Flow rate: A 1; B 2
Injection volume: 50
Detector: F ex 290 em >370 (filter)

CHROMATOGRAM

Retention time: 5.3
Internal standard: 3-(4-chlorophenyl)-1H-3,6-dimethylfuro[3,4-c]pyridin-7-ol (7.3)
Limit of quantitation: 50 ng/mL

KEY WORDS

plasma; column-switching; pharmacokinetics

REFERENCE

Antoniewicz,S.M.; Cook,J.A.; Brown,R.R. Determination of cicletanine in human plasma by high-performance liquid chromatography using automated column switching, *J.Chromatogr.*, **1992**, *573*, 93–98.

SAMPLE

Matrix: blood
Sample preparation: 2 mL Plasma + 100 μL 10 μg/mL IS in MeOH:water 10:90 + 7 mL diethyl ether, shake mechanically for 10 min, centrifuge at 1000 g for 5 min. Remove 5 mL of the organic layer and evaporate it to dryness under a stream of nitrogen at 40°, extract the aqueous layer with another 5 mL diethyl ether, remove 5 mL of the organic layer and add it to the residue from the first organic layer. Evaporate to dryness under a stream of nitrogen at 40°, reconstitute the residues in 200 μL MeCN:water 10:90, inject a 100 μL aliquot.

HPLC VARIABLES

Guard column: 5 × 4 4 μm Nova-Pak C18
Column: 100 × 8 4 μm Nova-Pak C18 Radial Pak
Mobile phase: MeCN:water 22:78 containing 0.02% isopropylamine and 0.085% phosphoric acid, pH 2.4 (Wash column with MeCN for 3 min at the end of each injection.)
Flow rate: 2.5
Injection volume: 100
Detector: UV 220

CHROMATOGRAM

Retention time: 10
Internal standard: (±)-2-methyl-3-hydroxy-4H,5H-5-methyl-(4'-chlorophenyl)isofuropyridine hydrochloride (17.5)
Limit of detection: 10 ng/mL

KEY WORDS

comparison with capillary electrophoresis; plasma; pharmacokinetics

REFERENCE

Pruñonosa,J.; Obach,R.; Diez-Cascón,A.; Gouesclou,L. Comparison of high-performance liquid chromatography and high-performance capillary electrophoresis for the determination of cicletanine in plasma, *J.Chromatogr.*, **1992**, *581*, 219–226.

SAMPLE

Matrix: blood
Sample preparation: 2 mL Whole blood or plasma + 2 mL buffer + 5 mL chloroform: isopropanol:n-heptane 60:14:26, shake gently horizontally for 10 min, centrifuge at 2800 g for 10 min. Remove the lower organic layer and evaporate it to dryness under vacuum at 45°, reconstitute the residue in 100 µL mobile phase, centrifuge at 2800 g for 5 min, inject a 50 µL aliquot of the supernatant. (Buffer was saturated ammonium chloride solution 25% diluted with water, adjusted to pH 9.5 with 25% ammonia solution.)

HPLC VARIABLES

Column: 300 × 3.9 4 µm NovaPack C18
Mobile phase: MeOH:THF:buffer 65:5:30 (Buffer was 0.68 g/L (10 mM (sic)) KH_2PO_4 adjusted to pH 2.6 with concentrated orthophosphoric acid.) (At the end of each session wash the column with water for 1 h and MeOH for 1 h, re-equilibrate for 30 min.)
Column temperature: 30
Flow rate: 0.8
Injection volume: 50
Detector: UV 220

CHROMATOGRAM

Retention time: 4.70
Limit of detection: <120 ng/mL

KEY WORDS

whole blood; plasma; interferences may occur—compounds(all of which are extracted) elute in this order tenoxicam; iproniazid; methocarbamol; methotrexate; caffeine; nialamide; colchicine; cytarabine; benzoylecgonine; acetaminophen; diazoxide; dacarbazine; sulfinpyrazole; flumazenil; sulpride; morphine; atenolol; toloxatone; terbutaline; albuterol; phenobarbital; ranitidine; tiapride; phenol; chlormezanone; aspirin; metformin; ritodrine; codeine; sultopride; amisulpride; naltrexone; lisinopril; benzocaine; nizatidine; nalorphine; mephenesin; naloxone; sotalol; carteolol; procainamide; carbamazepine; bromazepam; nalbuphine; nadolol; procarbazine; dihydralazine; omeprazole; strychnine; acebutolol; glutethimide; chlorpropamide; glipizide; triazolam; prazosin; flunitrazepam; clonazepam; metoclopramide; melphalan; estazolam; tolbutamide; ephedrine; clonidine; pindolol; clobazam; minoxidil; disopyramide; nitrazepam; dextromethorphan; tofisopam; zopiclone; debrisoquine; sulindac; alprazolam; cycloguanil; lorazepam; methaqualone; ketamine; piroxicam; metoprolol; nifedipine; quinine; mephentermine; prilocaine; pentazocine; oxazepam; tiaprofenic acid; quinidine; celiprolol; ajmaline; yohimbine; lidocaine; secobarbital; viloxazine; mepivacaine; meperidine; doxylamine; labetalol; temazepam; amodiaquine; benperidol; droperidol; hydroxychloroquine; zolpidem; ketoprofen; alminoprofen; cicletanine; moclobemide; chloroquine; cocaine; timolol; nomifensine; ticlopidine; acenocoumarol; vindesine; mexiletine; dipyridamole; trazodone; pipamperone; pyrimethamine; benazepril; vincristine; metapramine; chlordiazepoxide; oxprenolol; warfarin; clorazepate; flecainide; phencyclidine; thiopental; fenfluramine; metipranolol; triprolidine; na-

proxen; buprenorphine; verapamil; buspirone; tianeptine; midazolam; bupivacaine; carbinoxamine; loprazolam; cetirizine; chlorpheniramine; moperone; cibenzoline; medifoxamine; astemizole; vinblastine; nicardipine; bisoprolol; diltiazem; glibornuride; reserpine; aconitine; nitrendipine; diazepam; mianserin; ramipril; haloperidol; tetracaine; alprenolol; acepromazine; glibenclamide; chlorophenacinone; doxepin; nimodipine; diphenhydramine; cyclizine; histapyrrodine; phenylbutazone; demexiptiline; clozapine; proguanil; trifluperidol; medazepam; cyamemazine; bumadizone; suriclone; propranolol; acepromazine; dothiepin; dextromoramide; fenoprofen; dextropropoxyphene; loxapine; betaxolol; propafenone; promethazine; thioproperazine; methadone; amoxapine; quinupramine; opipramol; cyproheptadine; brompheniramine; mefenidramine; protriptyline; flurbiprofen; tetrazepam; zorubicin; prazepam; alimemazine; loperamide; imipramine; desipramine; levomepromazine; hydroxyzine; niflumic acid; penbutolol; fluvoxamine; pimozide; daunorubicin; indomethacin; maprotiline; tropatenine; etodolac; fluoxetine; amitriptyline; nortriptyline; tioclomarol; diclofenac; mefloquine; trimipramine; chlorambucil; lidoflazine; ibuprofen; floctafenine; alpidem; loratadine; chlorpromazine; clomipramine; carpipramine; thioridazine; fentiazac; clemastine; mefenamic acid; fluphenazine; prochlorperazine; penfluridol; bepridil; terfenadine; trifluoperazine

REFERENCE

Tracqui,A.; Kintz,P.; Mangin,P. Systematic toxicological analysis using HPLC/DAD, *J.Forensic Sci.*, **1995**, *40*, 254–262.

SAMPLE

Matrix: blood, urine

Sample preparation: Add 1 mL whole blood or urine to Toxi-Tube A (Toxi-Lab, Irvine CA), add 3 mL water, mix by gentle inversion for 5 min, centrifuge at 1500 g for 5 min. Remove the organic layer and evaporate it to dryness under a stream of nitrogen at 40°, reconstitute the residue with 50 µL MeCN:water 50:50, vortex for 10 s, centrifuge at 7500 g for 2 min, inject a 10 (urine) or 30 (blood) µL aliquot. (The detector wavelength shown is the wavelength of maximum absorbance. This will not necessarily be the optimal wavelength for the separation. Multiple wavelengths from 200-350 nm can be scanned using a diode-array detector. Otherwise, 220 nm may be a reasonable choice for initial work. Matrix may interfere.)

HPLC VARIABLES

Guard column: 20 mm long Symmetry C18

Column: 250 × 4.6 5 µm Symmetry C8 (Waters)

Mobile phase: Gradient. A was 50 mM pH 3.8 sodium phosphate buffer. B was MeCN. A:B 85:15 for 6.5 min, 65:35 for 18.5 min, 20:80 for 3 min (step gradient), re-equilibrate at initial conditions for 7 min.

Column temperature: 30

Flow rate: 1 for 6.5 min, to 1.5 over 18.5 min, maintain at 1.5 for 3 min (re-equilibrate at 1.5 mL/min)

Injection volume: 10-30

Detector: UV 200.5

CHROMATOGRAM

Retention time: 13.808

KEY WORDS

whole blood

REFERENCE

Gaillard,Y.; Pépin,G. Use of high-performance liquid chromatography with photodiode-array UV detection for the creation of a 600-compound library. Application to forensic toxicology, *J.Chromatogr.A*, **1997**, *763*, 149–163.

SAMPLE

Matrix: urine

Sample preparation: 200 μL Urine + 20 μL 10 μg/mL methylcicletanine, adjust pH to 5.2 with 50 μL 1 M acetate buffer, add 2 mL hexane:ether 80:20, vortex, centrifuge, repeat extraction. Combine the organic layers and evaporate them to dryness under a stream of nitrogen, reconstitute the residue in 50 μL MeOH, inject a 20 μL aliquot. (Hydrolyze sulfate conjugates by heating 200 μL urine with 400 μL 1 M HCl at 37° for 12 h. Hydrolyze glucuronide conjugate with β-glucuronidase (E.C.3.21.31, Helix pomatia type H-1, Sigma) at 37° at pH 5.2 for 12 h.)

HPLC VARIABLES

Column: 250 × 4 5 μm ChiraDex (Merck)

Mobile phase: MeOH:water 30:70 containing 0.1% triethylammonium chloride, pH adjusted to 4.0

Column temperature: 30

Flow rate: 1

Injection volume: 20

Detector: UV 220

CHROMATOGRAM

Internal standard: methylcicletanine

Limit of detection: 1 μg/mL

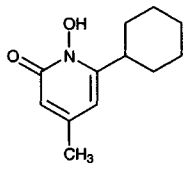
KEY WORDS

chiral; rat

REFERENCE

Vistelle,R.; Lamiable,D.; Morin,E.; Trenque,T.; Kaltenbach,M. Urinary excretion of cicletanine in the rat. Stereochemical aspects, *Drug Metab.Dispos.*, **1995**, *23*, 988–992.

Ciclopirox



Molecular formula: $C_{12}H_{17}NO_2$
Molecular weight: 207.27
CAS Registry No.: 29342-05-0, 41621-49-2 (olamine)
Merck Index: 2325

SAMPLE

Matrix: blood
Sample preparation: Condition an Adsorbex CN SPE cartridge (E. Merck) with 1 mL MeCN. 1 mL Plasma + 1 mL 1/15 M pH 5.0 phosphate buffer (KH_2PO_4 and Na_2HPO_4) + 10 µL 40 IU/mL β-glucuronidase, incubate at 37° for 24 h, add 500 µL 200 mM NaOH, add 200 µL dimethyl sulfate (CAUTION! Highly Toxic!), heat at 37° for 20 min, add 200 µL triethylamine, add 10 mL n-hexane, extract. Add 8 mL of the organic phase to the SPE cartridge, wash with 1 mL toluene, aspirate to dryness under reduced pressure for 3 min, elute with 350 µL mobile phase, evaporate eluate at 40° under nitrogen, dissolve residue in 80 µL mobile phase, inject a 50 µL aliquot.

HPLC VARIABLES

Guard column: 5 µm LiChrospher 100 RP18 in a LiChroCART 4-4
Column: 125 × 4 5 µm LiChrospher 100 RP18
Mobile phase: MeCN:water 40:60
Flow rate: 1
Injection volume: 50
Detector: UV 304

CHROMATOGRAM

Retention time: 7.6
Limit of detection: 15 ng/mL

KEY WORDS

plasma; SPE; rabbit; derivatization; pharmacokinetics

REFERENCE

Coppi,G.; Silingardi,S. HPLC method for pharmacokinetic studies on ciclopirox olamine in rabbits after intravenous and intravaginal administrations, *Farmaco*, **1992**, *47*, 779–786.

SAMPLE

Matrix: formulations
Sample preparation: Dissolve 1 g cream, lotion, or shampoo in 100 mL MeOH:water 70:30 with sonication, centrifuge, filter (0.45 µ), inject a 10 µL aliquot.

HPLC VARIABLES

Column: 250 × 4 5 µm Purospher RP-18 endcapped (Merck)
Mobile phase: MeCN:water containing 20 mM phosphoric acid and 500 µM disodium EDTA 68:32
Flow rate: 1
Injection volume: 10
Detector: UV 305

CHROMATOGRAM

Retention time: 4.25

OTHER SUBSTANCES

Simultaneous: metabolites, octopirox

KEY WORDS
cream; lotion; shampoo

REFERENCE
Gagliardi,L.; Multari,G.; Cavazzutti,G.; De Orsi,D.; Tonelli,D. HPLC determination of ciclopirox, octopirox, and pyrithiones in pharmaceuticals and antidandruff preparations, *J.Liq.Chromatogr.Rel.Technol.*, **1998**, *21*, 2365–2373.

SAMPLE
Matrix: formulations
Sample preparation: Foam. Weigh out an amount equivalent to about 10 mg ciclopirox, add 2 mL 1 M NaOH, add 30 μL methyl iodide, vortex, keep in an ice bath for 10 min, add 30 μL 25% ammonium hydroxide, dilute to 50 mL with MeCN:water 1:1, inject a 0.2 μL aliquot. Powder. Weigh out an amount equivalent to about 2 mg ciclopirox, add 2 mL MeCN:water 1:1, add 1 mL 1 M NaOH, add 20 μL methyl iodide, vortex, keep in an ice bath for 10 min, add 20 μL 25% ammonium hydroxide, dilute to 50 mL with MeCN:water 1:1, inject a 0.2 μL aliquot.

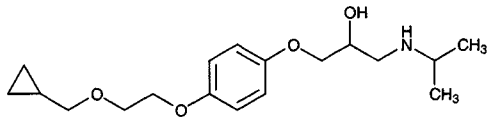
HPLC VARIABLES
Column: 150 × 0.33 5 μm 300 Å DeltaPak RP-18 fused silica capillary (Fusica, LC Packings)
Mobile phase: MeCN:water 50:50
Column temperature: 20
Flow rate: 0.01
Injection volume: 0.2
Detector: UV 300

CHROMATOGRAM
Retention time: 2
Limit of detection: 10 ng

KEY WORDS
foam; powder; capillary HPLC; derivatization

REFERENCE
Belliardo,F.; Bertolino,A.; Brandolo,G.; Lucarelli,C. Micro-liquid chromatography method for the determination of ciclopiroxolamine after pre-column derivatization in topical formulations, *J.Chromatogr.*, **1991**, *553*, 41–45.

Cicloprolol



Molecular formula: $C_{18}H_{29}NO_4$
Molecular weight: 323.44
CAS Registry No.: 94651-09-9, 63659-12-1 (without (±)-definition), 63686-79-3 (HCl)
Lednicer No.: 4 25

SAMPLE
Matrix: solutions

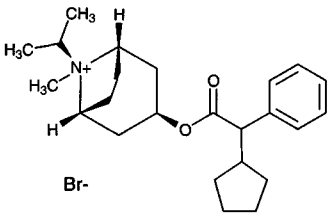
HPLC VARIABLES
Column: 150 × 4.6 12 μm 1-myristoyl-2-[(13-carboxyl)-tridecoyl]-sn-3-glycerophosphocholine chemically bonded to silica (Regis)
Mobile phase: MeCN:100 mM pH 7.0 phosphate buffer 20:80
Flow rate: 1
Detector: UV 254

CHROMATOGRAM
Retention time: k' 10.28

OTHER SUBSTANCES
Also analyzed: acebutolol, alprenolol, antazoline, atenolol, betaxolol, bisoprolol, bopindolol, bupranolol, carteolol, chloropyramine, chlorpheniramine, cicloprolol, cimetidine, cinnarizine, cirazoline, clonidine, dilevalol, dimethindene, diphenhydramine, doxazosin, esmolol, famotidine, isothipendyl, ketotifen, metiamide, metoprolol, moxonidine, nadolol, naphazoline, nifenalol, nizatidine, oxprenolol, pheniramine, phentolamine, pindolol, pizotyline (pizotifen), practolol, prazosin, promethazine, propranolol, pyrilamine (mepyramine), ranitidine, roxatidine, sotalol, tiamenidine, timolol, tramazoline, tripelennamine, triprolidine, tymazoline, UK-14,304

REFERENCE
Kaliszan,R.; Nasal,A.; Turowski,M. Binding site for basic drugs on α_1-acid glycoprotein as revealed by chemometric analysis of biochromatographic data, *Biomed.Chromatogr.*, **1995**, *9*, 211–215.

Ciclotropium bromide



Molecular formula: $C_{24}H_{36}BrNO_2$
Molecular weight: 450.46
CAS Registry No.: 85166-20-7

SAMPLE

Matrix: blood, urine

Sample preparation: Plasma. 4 mL Plasma + 5.5 mL chloroform + 500 µL reagent A + 250 µL 1 M HCl, shake vigorously for 5 min, centrifuge at 10° at 2500 g for 30 min. Remove a 4 mL aliquot of the organic layer and add it to 2.4 mL 100 mM HCl, shake for 15 min, centrifuge at 20° at 2500 g for 10 min. Remove a 2 mL aliquot of the aqueous layer and add it to 1 mL 1 M NaOH, heat at 140-5° for 90 min (to hydrolyse ciclotropium), cool to room temperature, add 500 µL reagent A, adjust pH to 8.5-9.5 with 5 M HCl, add 2.6 mL chloroform, shake for 15 min, centrifuge at 20° at 2500 g for 10 min. Remove a 2 mL aliquot of the chloroform layer and add it to 1.4 mL 100 mM HCl, shake for 15 min, centrifuge at 20° at 2500 g for 10 min. Remove a 1 mL aliquot of the aqueous layer and add it to 1 mL MeOH, evaporate to dryness under reduced pressure, add three 3 mL portions of MeOH and evaporate to dryness each time, take up the residue in 200 µL 10 mg/mL flunoxaprofen chloride in MeCN (freshly prepared), heat at 110° for 15 min, evaporate to dryness under reduced pressure, add 1 mL ethyl acetate, add 1.3 mL 10 mM HCl, shake vigorously for 15 min, centrifuge at 20° at 2500 g for 10 min, discard the organic layer, wash the aqueous layer twice more with ethyl acetate, evaporate the aqueous layer to dryness under reduced pressure, reconstitute with 100 µL MeCN:MeOH: water 1:1:1, inject a 20 µL aliquot. Urine. 1 mL Urine + 100 µL 1 µg/mL IS in water + 2 mL 0.5 M NaOH, heat at 140-5° for 90 min (to hydrolyse ciclotropium), cool to room temperature, add 500 µL reagent B, adjust pH to 8.5-9.5 with 5 M HCl, add 2.6 mL chloroform, shake for 15 min, centrifuge at 20° at 2500 g for 10 min. Remove a 2 mL aliquot of the chloroform layer and add it to 1.4 mL 100 mM HCl, shake for 15 min, centrifuge at 20° at 2500 g for 10 min. Remove a 1 mL aliquot of the aqueous layer and add it to 1 mL MeOH, evaporate to dryness under reduced pressure, add three 3 mL portions of MeOH and evaporate to dryness each time, take up the residue in 200 µL 10 mg/mL flunoxaprofen chloride in MeCN (freshly prepared), heat at 110° for 15 min, evaporate to dryness under reduced pressure, add 1 mL ethyl acetate, add 1.3 mL 10 mM HCl, shake vigorously for 15 min, centrifuge at 20° at 2500 g for 10 min, discard the organic layer, wash the aqueous layer twice more with ethyl acetate, evaporate the aqueous layer to dryness under reduced pressure, reconstitute with 500 µL MeCN:MeOH: water 1:1:1, inject a 20 µL aliquot. (Prepare reagent A by mixing 100 mg dipicrylamine, 600 mg anhydrous sodium carbonate, and 10 mL water. Prepare reagent B by mixing 35 mg dipicrylamine and 10 mL 100 mM NaOH. Prepare dipicrylamine as follows (Caution! Dipicrylamine is potentially explosive and highly toxic, store moistened with 50% water!). Add 50 g 2,4-dinitrodiphenylamine to 420 g nitric acid (36° Bé. 52%, d = 1.33) heated to 62° over 2 h, heat at 62-90° for another 3 h, cool, filter, wash the product until it is free of acid, dry to obtain 2,2',4,4'-tetranitrodiphenylamine as a yellow solid (mp 187.4°). Add 50 g tetranitrodiphenylamine over 1 h to 500 g of a mixture of equal parts 92% sulfuric acid and 93% nitric acid at room temperature, after 4.5 h add to a large volume of ice water, filter, recrystallize the product from acetone to obtain dipicrylamine (2,2',4,4',6,6'-hexanitrodiphenylamine) as yellow crystals (mp 242.9°) (J.Am.Chem.Soc. 1919, 41, 1013). Prepare flunoxaprofen chloride as follows. Dissolve 1 mmole flunoxaprofen in 25 mL toluene, add a trace of DMF (J.Chromatogr. 1990, 528, 55), add 2.5 mL thionyl chloride, reflux for 30 min, remove solvent by evaporation, dry the residue under vacuum over KOH, recrystallize from dichloromethane (mp 73°) (J.Chromatogr. 1988, 427, 131).)

HPLC VARIABLES

Column: 150 × 3.9 5 µm Suplex pkb-100 (Supelco)

Mobile phase: MeCN:water 55:45 containing 1 mL/L 50% phosphoric acid and 0.6 g/L dodecyl sulfate.
Flow rate: 1
Injection volume: 20
Detector: F ex 310 em 365

CHROMATOGRAM

Retention time: 8.0
Internal standard: N-butyltropinium (11.1)
Limit of detection: 0.5 ng/mL (plasma), 10 ng/mL (urine)

KEY WORDS

derivatization; plasma; pharmacokinetics

REFERENCE

Liebmann,B.; Henke,D.; Spahn-Langguth,H.; Mutschler,E. Determination of the quaternary compound ciclotropium in human biological material after hydrolysis and derivatization with the fluorophor flunoxaprofen chloride, *J.Chromatogr.*, **1991**, *572*, 181–193.